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Review article

DNA polymerase β deficiency promotes the occurrence of esophageal precancerous lesions in mice

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Abstract

Esophageal mucosa undergoes mild, moderate, severe dysplasia, and other precancerous lesions and eventually develops into carcinoma in situ, and understanding the developmental progress of esophageal precancerous lesions is beneficial to prevent them from developing into cancer. DNA polymerase β (Polβ), a crucial enzyme of the base excision repair system, plays an important role in repairing damaged DNA and maintaining genomic stability. Abnormal expression or deletion mutation of Polβ is related to the occurrence of esophageal cancer, but the role of Polβ deficiency in the esophageal precancerous lesions is still unclear. Here, esophageal mucosa Polβ-knockout mice were used to explore the relationship of Polβ deficiency with esophageal precancerous lesions. First, we found the degree and number of esophageal precancerous lesions in Polβ-KO mice were more serious than those in Polβ-Loxp mice after N-nitrosomethylbenzylamine (NMBA) treatment. Whole exome sequencing revealed that deletion of Polβ increased the frequency of gene mutations. Gene expression prolife analysis showed that the expression of proteins correlated to cell proliferation and the cell cycle was elevated in Polβ-KO mice. We also found that deletion of Polβ promoted the proliferation and clone formation as well as accelerated cell cycle progression of human immortalized esophageal epithelial cell line SHEE treated with NMBA. Our findings indicate that Polβ knockout promotes the occurrence of esophageal precancerous lesions.

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Keywords: Polβ, NMBA, Esophageal precancerous lesion

Introduction

DNA polymerase beta (Polβ) is a member of the DNA polymerase X family, which encodes a single-stranded polypeptide with a molecular weight of 39 KD and plays a crucial role in the base excision repair (BER) system [1,

Abbreviations: BER, base excision repair; CCK8, Cell counting kit-8; COSMIC, the Catalogue of Somatic Mutations in Cancer database; GEPIA, the Gene Expression Profiling Interactive Analysis database; Polβ, DNA polymerase beta; NMBA, N-nitrosomethylbenzylamine; SHEE, the human immortalized esophageal epithelial cell line.

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2] . Polβ has two independent domains: a lyase domain at the N-terminus and a polymerase domain at the C-terminus. The two domains determine the different functions of Polβ in the BER process [3, 4]. The BER pathway is activated when bases in the DNA double-strand are damaged by endogenous or exogenous factors. In short patch BER, the lyase domain of Polβ recognizes and removes the 5′dRP and then inserts the correct base to repair the damaged site. In long patch BER, after 2 to 13 nucleotide strand displacement synthesis mediated by Polβ and/or Polδ/ε, the 5′-DNA flap is removed by FEN1 and the gap is closed by DNA ligase LGI1 to complete the repair [5].

As a crucial enzyme of the BER pathway, Polβ is essential to repair damaged DNA and maintain genomic stability. When BER fails due to abnormal expression or mutation of Polβ, damaged DNA cannot be repaired and gene mutations accumulate, which may lead to uncontrolled cell growth and malignant tumor formation. Some studies have shown that a lack of Polβ reduces BER efficiency and sensitizes cells to alkylation or oxidants agents [6, 7]. In the nervous system, selective inactivation of Polβ is related to the occurrence of medulloblastoma [8]. Additionally, mutations in Polβ have been detected in various cancers. For example, PolβR137Q, PolβP242R, and PolβR152C have been identified in human somatic cells, which weaken the activity of Polβ and BER efficiency, and may finally promote genomic instability and cancer development [9-11]. Dong et al. found that the high frequency deletion mutations of Polβ (35%) was detected in human esophageal cancer samples, which indicated there is a relationship between Polβ and esophageal cancer [12]. However, the mechanism of Polβ deficiency in promoting the occurrence and development of esophageal precancerous lesions remains unclear and animal models used to explore the relationship of Polβ deletion and esophageal tumorigenesis are rare.

In this study, we established esophageal mucosal Polβ-deficient mice using the Cre-loxP-mediated recombination system, which were treated for 5 wk with 2 mg/kg NMBA, a nitrosamine that has been used to induce esophageal cancer and esophageal precancerous lesions in rats and mice [13-15]. Hematoxylin and eosin staining showed that the degree and number of lesions in Polβ-KO mice were more severe than those in Polβ-Loxp mice. And the whole exon sequencing of esophageal mucosa in two group showed that the gene mutation frequency of Polβ-KO mice was higher than that of Polβ-Loxp mice. Next, we harvested esophageal epithelial tissue for gene expression profile and GO/KEGG enrichment analyses, the results indicated that the expression of PLK1, PI3K, AKT, ERK, and Ki67, which are related to cell cycle and cell proliferation, was increased in Polβ-KO mice. In addition to observations at the cellular level, NMBA treatment of immortalized esophageal mucosal epithelial cell line SHEE with Polβ knockdown enhanced cell proliferation and colony formation. In general, Polβ deficiency facilitates the occurrence of esophageal precancerous lesions induced by NMBA, which may be related to promotion of gene mutations and accelerating malignant proliferation of cells.

Materials and methods

Cell culture and reagents

The human immortalized esophageal epithelial cell line SHEE was provided by Professor Enmin Li (Institute of Cancer Pathology, Shantou University Medical College, Guangdong, China). The 293T cell line was purchased from the National Collection of Authenticated Cell Cultures (Beijing, China). All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. N-nitrosomethylbenzylamine (NMBA) was purchased from East China University of Science and Technology (Shanghai, China) and the purity was 98% as determined by high performance liquid chromatography.

Model establishment and animal experiments

Animal experiments were approved by the Research Ethics Committee of Zhengzhou University and conformed to their guidelines. Mice were housed in a specific pathogen-free breeding barrier under a 12 h light/dark cycle and fed *ad libitum*. Pol$\beta^{flox/flox}$ mice were purchased from the Jackson Laboratory (USA) and ED-L2-CRE$^{+/-}$ mice were a gift from the National Cancer Institute (USA) mouse library. Pol$\beta^{flox/flox}$ mice were crossed with ED-L2-CRE$^{+/-}$ mice to obtain heterozygous offspring: Pol$\beta^{flox/wild}$, ED-L2-CRE$^{+/-}$. Then, Pol$\beta^{flox/wild}$, ED-L2-CRE$^{+/-}$ mice were crossed with each other to generate Pol$\beta^{flox/flox}$, ED-L2-CRE$^{+/-}$ mice (Polβ-KO mice) and Pol$\beta^{flox/flox}$, ED-L2-CRE$^{-/-}$ mice (Poclβ-Loxp mice). Mice were divided into Polβ-KO and Polβ-Loxp groups at 7–9 wk of age. All mice were hypodermically injected with 2 mg/kg NMBA dissolved in sterile water three times a wk for 5 wk and then monitored. At wk 31, five mice in each group were randomly selected for euthanasia and esophageal tissues were extracted to determine whether there were any lesions. At wk 53, all mice were euthanized. Esophageal tissues were harvested and one half was to extract DNA, RNA, or protein and the other half was fixed in 10% neutral buffered formalin.

PCR assays

DNA was extracted from the tail using tissue lysate buffer (1 M Tris-HCl, pH 8.5, 0.5 M EDTA, pH 8.0, 10% SDS, 5 M NaCl, and 20 mg/ml protease K) at 21 d of age. The genotype of mice was determined by PCR and agarose gel electrophoresis. Tail DNA was amplified under the following conditions: 94°C for 3 min, 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and then final extension at 72°C for 5 min to acquire a 400 bp floxed Polβ fragment or 94°C for 3 min, 35 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then final extension at 72°C for 5 min to acquire a 199 bp CRE fragment.

The primes were as follows: Polβ forward, 5′-TTTCTTAGCTT-CCGCTCCG-3′ and reverse, 5′-CAGGCGATCCACAAGTACAAT-3′; L2-CRE forward, 5′-ACCAGCCAGCTATCAACTCG-3′ and reverse, 5′–TTACATTGGTCCAGCCACC-3′; reference gene forward, 5′-CTAGG-CCACAGAATTGAAAGATCT-3′ and reverse, 5′-GTAGGTGGAAATT-CTAGCATCATCC-3′.

Hematoxylin and eosin staining

Esophageal tissues fixed with 10% formalin and embedded in paraffin were cut in 4-μm thick sections. The sections were stained with HE and scanned using a TissueFAXS (TissueGnostics GmbH, Vienna, Austria). The pathological grade of esophageal was divided into five histological categories including normal epithelium, epithelial hyperplasia, mild dysplasia, moderate dysplasia, and severe dysplasia. Normal esophageal epithelial cells are usually arranged in order with a thickness of one to two cells. Hyperplasia consists of slight thickening of the basal cell and keratin layers. Dysplasia is characterized by disorder of epidermal proliferation, increased cell atypia, and thickening of the keratin layer. Dysplasia includes mild, moderate and severe dysplasia characterized by thickening of cell atypia by less than 1/3, 1/3 to 2/3, and more than 2/3 in overall esophageal epithelium. The histological category standard of esophagus is classified in accordance with the Philip R Taylor and Gray D. Stoner classification criteria [16].

Immunohistochemical staining

Esophageal tissues sections (4 μm thick) were heated at 65°C for 2 h and then soaked in xylene and ethanol to remove the paraffin. Antigen retrieval was performed by microwaving for 10 min in citrate buffer (pH 6.0). Tissues were then incubated with 3% H_2O_2 for 5 min. Primary anti-Pol and anti-PLK1 antibodies were applied at 4°C overnight. Then, the tissues were

incubated with an HRP-IgG secondary antibody at 37°C for 15 min. Tissues were stained with DAB and hematoxylin. After dehydration, the sections were covered with neutral resin, were scanned with the TissueFAXS, and analyzed using HistoQuest 4.0 software (TissueGnostics GmbH).

Affymetrix Genechip analysis

Esophageal mucosa of three mice from each group was used to extract total RNA on ice to produce cDNA by reverse transcription. Subsequently, the cDNA labeled with biotin was hybridized to a mouse oligonucleotide probe array (U430, Affymetrix, Santa Clara, CA, USA) to acquire the gene expression profile. Genes with significant differences were screened in accordance with $|Fold\ Change|>1.5$ and $P\text{-}value < 0.05$ as the criteria and subjected to GO annotation and KEGG signaling pathway analyses.

Whole exome sequencing analysis

Qualified genomic DNA from esophageal mucosa of the two groups was fragmented by Covaris technology (Covaris, Inc, USA) [17]. Subsequently, the DNA was used to construct a high quality library for exome capture, and sequencing. The library was hybridized to a mouse exome array for enrichment and nonhybridized fragments were washed out. After PCR amplification and qualifying the captured library, sequencing was performed on the HiSeq2000 platform (Illumina, USA). Raw image files were produced by calling with default parameters and sequences were generated by paired-end reads. All genome variants, including SNP and In-Del were detected and then subjected to a series of annotation and advanced analyses.

Western blotting

RIPA lysis buffer (100 mM phenylmethanesulfonyl fluoride, 500 mM NaF, 200 mM NaVO4, and protease inhibitor) was used to extract proteins from esophageal tissues and SHEE cells. The protein concentration was measured using a Bicinchoninic acid protein assay kit (Beyotime, Shanghai, China). Proteins (30 to 50 μg) were separated by 10% SDS-PAGE and then transferred to a PVDF membrane at 90 V for 2 h. Subsequently, the membrane was blocked with 5% dry nonfat milk at room temperature for 1 h. Primary antibodies were applied at 4°C overnight and then a secondary antibody (anti-rabbit HRP or anti-mouse HRP, 1:10000) was applied at room temperature for 2 h. Finally, protein bands were visualized using enhanced chemiluminescence detection reagent (GE Healthcare Life Science) and their densities were calculated by ImageJ software. The primary antibodies were against Polβ, PLK1 (1:1000, Abcam, USA), Tubulin and Actin (1:3000, ZSGB-BIO, China).

Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) and reverse transcription was performed to generate cDNA with a PrimeScript™ RT Reagent Kit (Takara, Japan). Then, cDNA samples were amplified using SYBR Green PCR Master Mix (Takara, Japan). GAPDH was used as an internal control. Gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: Polβ forward, 5′-TTTCTTAGCTTCCGCTCCG-3′ and reverse, 3′-CAGGCGATCCACAAGTACAAT-5′; PLK1 forward, 5′-TCGAGATCTCAGACGCAGAC-3′ and reverse, 5′- GTCAGTGCCTTCCTCCTCTT-3′; *GAPDH* forward, 5′-AGGTCGGTGTGAACGGATTTG-3′ and reverse, 3′-TGTAGACCATGTAGTTGAGGTCA-5′.

Lentivirus transduction

Lentiviral construct pLKO.1 for shRNA was used to produce pLKO.1-scramble shRNA (sh-mock) and pLKO.1-polβ-shRNA plasmids (shPolβ#1, shPolβ#2, and shPolβ#3, GE, USA). After the plasmids and packaging vector (psPAX2, pMD2.G) were mixed with 200 μl jet buffer and 4 μl jet prime for 15 min, the DNA was transfected into 293T cells. The medium was replaced after 4 h and lentiviral particles were collected at 24 and 48 h. SHEE cells were cultivated in medium containing lentiviral particles and 8 μg/mL polybrene. After 24 h, puromycin (1 μg/mL) was used to select the cells. Then, the cells were collected and protein was extracted to detect the expression of Polβ by western blotting. shRNA sequences were as follows:

shPolβ#1 mature antisense, 5′-TTTGTCTCACCCTTTGACAGG-3′;
shPolβ#2 mature antisense, 5′-AAAGTTTGCGAGTTCTGTGAG-3′;
shPolβ#3 mature antisense, 5′-TTCATCTACAAACTTCCTTGC-3′.

Immunofluorescence

Cells were seeded in 12-well plates at a density of 1.5×10^4 per well and treated with 10 μM NMBA for 48 h. Cells were fixed in 4% paraformaldehyde for 30 min and then permeabilized with 0.2% Triton X-100 in PBS. Non-specific binding sites were blocked with 1% BSA in TBST. Sections were incubated with primary antibodies at 4°C for overnight and then with anti-rabbit FITC-labelled or anti-mouse TRITC-labelled secondary antibodies at room temperature for 2 h. Subsequently, the cells were counterstained with DAPI, mounted, and stored at -20°C while protected from light until analysis. The fluorescence of cells was observed and imaged using an In Cell Analyzer 6000 (GE, USA).

Cell counting kit-8 (CCK8) assay

Cells treated with 50 μM NMBA for 10 d were seeded in 96-well plates at a density of 2.5×10^3 cells per well and incubated in 37°C with 5% CO_2 overnight. After adding CCK8 reagent (TargetMol, USA) to each well at 0, 24, 48, 72, and 96 h, the cells were incubated in 37°C in the dark for 2 h and then the OD value was measured at 450 nm by a microplate reader (Thermo, USA).

Anchorage-independent cell growth assay

The lower basal medium consisted of 40% 2 × BME, 1% glutamine, 0.1% gentamicin, 10% FBS, 9% sterile water, 40% agarose (1.25%), 50 μM NMBA. and 10 μg/mL EGF. The medium was added to a six-well plate at 3 mL per well and then incubated for 2 h. Cells (8×10^3) treated with 50 μM NMBA for 10 d were resuspended in the upper basal medium and seeded at 1 mL per well. The upper basal medium consisted of 45% 2 × BME, 1% glutamine, 0.1% gentamicin, 10% FBS, and 45% sterile water. The cells were cultured at 37 °C with 5% CO_2 for 10 d. Colonies were scanned and counted using the In Cell Analyzer 6000 (GE, USA).

Colony formation assay

Cells (500 cells per well) were seeded in 6-well plates and then treated with 50 μM NMBA for 10 d. The cells were washed twice with PBS fixed with 4% paraformaldehyde for 30 min, and then stained with 0.1% crystal violet for 30 min at 37°C. Then, images were obtained and the number and size of colonies were analyzed.

Cell cycle analysis

Cells (3×10^4) were seeded in 60-mm dishes and incubated overnight. The cells were cultured in serum-free medium for 24 h and then in medium

containing NMBA (50 μM) for 24 h. The cells were washed with PBS and fixed with precooled 70% ethanol at 4°C overnight. Then, RNase (50 μg/ml) was applied at room temperature to remove RNA. Subsequently, the cells were stained with propidium iodide (50 μg/ml) for 30 min and the cell cycle distribution were analyzed by using FACScan flow cytometer (BD FACSCanto) and FlowJo V10 software.

Results

Successful establishment of esophageal mucosal Polβ-specific knockout mice

Pol$\beta^{flox/flox}$ and ED-L2-Cre mice were crossed to obtain F1 heterozygous mice (Pol$\beta^{flox/wild}$, ED-L2-CRE$^{+/-}$). Subsequently, F1 heterozygous mice were mated with each other to acquire Polβ knockout mice and the genotype was confirmed by extracted DNA from mice tail. The genotypes of Polβ-KO and Polβ-Loxp mice were Pol$\beta^{flox/flox}$, ED-L2-CRE$^{+/-}$ and Pol$\beta^{flox/flox}$, ED-L2-CRE$^{-/-}$, respectively (Fig. 1A and B). Next, to confirm knockout of Polβ, total RNA of esophageal mucosa tissue and esophageal muscle layer tissue of the two groups was extracted to measure the mRNA level of Polβ. The results showed that the Polβ mRNA level in esophageal mucosa in Polβ-KO mice was obviously reduced compared with that in Polβ-Loxp mice (Fig. 1C). Moreover, we measured the protein expression level of Polβ in esophageal mucosa by western blotting. Similarly, the protein level of Polβ in Polβ-KO mice was obviously decreased (Fig. 1D). To determine whether knockout of Polβ affected the growth and development of mice, we monitored their weight from 3 to 36 d of age and found that the weight of Polβ-KO mice had no significant difference compared with that of Polβ-Loxp mice (Fig. 1E). Additionally, we found no abnormal changes in the morphology and structure of esophageal epithelial tissue (Fig. 1F). These results showed that Polβ knockout mice were established successfully.

Loss of Polβ increases the incidence of esophageal precancerous lesions after NMBA treatment

As a DNA damage inducer, NMBA has been used to induce esophageal precancerous lesions and the formation of forestomach papillomas in mice [15, 18]. In this study, we investigated whether loss of Polβ increased the incidence of esophageal precancerous lesions after subcutaneous injection of NMBA (2 mg/kg) 3 times a wk for 5 wk. At 31 wk, 5 mice were selected to extract the esophagus for HE staining after euthanasia. In accordance with the diagnostic criteria of esophageal precancerous lesions, severe dysplasia was found in Polβ-KO mice and the total number of lesions was more than that in Polβ-Loxp mice, while Polβ-Loxp mice only showed mild dysplasia (Fig. 2D and Supplementary Table 1). Typical images of esophageal precancerous lesions are shown in Fig. 2C. At 53 wk, severe dysplasia of esophageal mucosa appeared in both groups of mice, but the probabilities of simple hyperplasia, mild dysplasia, moderate dysplasia, and sever dysplasia in Polβ-KO mice were higher than those in Polβ-Loxp mice (Fig. 2F and Supplementary Table 1). Additionally, the total number of lesions in Polβ-KO mice was more than that in Polβ-Loxp mice (Fig. 2G). No significant difference in the body weight of the two groups was observed (Fig. 2B). These results indicated that the absence of Polβ increased the incidence of esophageal precancerous lesions after NMBA treatment.

Loss of Polβ increases the frequency of gene mutations

DNA Polβ is a DNA repair enzyme that repairs damaged DNA through base excision repair [19]. In this study, the degree of esophageal precancerous lesions in Polβ-KO mice was more serious than that in Polβ-Loxp mice. To examine whether deletion of Polβ led to accumulation of DNA damage, we extracted the esophageal mucosa for whole exome sequencing to detect gene mutations. A total of 539 gene mutations were detected in Polβ-KO mice. Among them, mutations located in exon regions included 34 missense SNVs, 10 synonymous SNVs, one stop-gain, and seven unknown mutations (Fig. 3A). Missense mutation—replacement of bases in the genome—is the most common type of gene mutation, which may cause changes in the structure or function of gene-encoded products and then affect the normal operation of cells and cause tumorigenesis. The most common substitution in our data was transition C, G>T, A, which was consistent with the most common G>A mutation known in the Catalogue of Somatic Mutations in Cancer (COSMIC) database for esophageal cancer (Fig. 3B). Additionally, we compared these genes with the COSMIC database and found that ap1g2, adamts12, sulf1, ank2f1, ugt1a, necap2, tshz3, eppk1, and pkd1 are known driver genes of esophageal cancer (Table 1). We also found that adamts12, sulf1, eppk1, and sp110 were highly expressed in esophageal cancer by comparison with the Gene Expression Profiling Interactive Analysis (GEPIA) database (Table 1 and Supplementary Fig. 1). These data suggested that the loss of Polβ promoted genome instability and increased the frequency of gene mutations.

Analysis of gene expression profile

To better explore how Polβ knockout promoted the occurrence and development of esophageal precancerous lesions, we harvested esophageal mucosal tissue from Polβ-Loxp mice and Polβ-KO mice treated with NMBA to detect mRNA expression changes by gene expression profile analysis. In accordance with $|fold\ change| > 1.5$ and $P\text{-}value < 0.05$ for significant differences, 504 significant differentially expressed genes were selected and 208 genes were upregulated and 296 genes were downregulated (Fig. 4A). GO annotation and KEGG pathway enrichment analysis were carried out using these genes with significant differential expression, and we found that the genes participated in many important biological processes including cell proliferation and the cell cycle (Figs. 4B–E). In the KEGG pathway enrichment analysis, glucagon and insulin signaling pathways were significantly enriched and the levels of PIK3R5 and PRKAG2, which regulate cell proliferation, were increased in the pathways (Fig. 4E). Additionally, we measured the expression of Ki67, PI3K, p-AKT, p-ERK, and p-PLK1 related to cell proliferation in esophageal mucosal by immunohistochemistry. The results indicated that expression of these proteins was increased in Polβ-KO mice (Fig. 4G). These results suggested the loss of Polβ led to progression of esophageal malignancy by affecting cell proliferation and cell cycle-related proteins.

Loss of Polβ promotes proliferation of esophageal epithelial cells after NMBA treatment

We confirmed that loss of Polβ in esophageal mucosa increased the severity of precancerous lesions after NMBA treatment. To confirm whether Polβ deficiency promoted malignant proliferation of cells, the human immortalized esophageal epithelial cell line SHEE was used to establish Polβ knockdown cell lines and transfection efficiency was detected by western blotting (Fig. 5A). Subsequently, cells were treated with 50 μM NMBA for 10 d and CCK8 assays were used to assess cell proliferation. The results showed that knockdown of Polβ promoted cell proliferation after NMBA treatment (Fig. 5B). Moreover, colony formation and anchored growth of SHEE cells with Polβ knockdown were significantly enhanced compared with the control (Figs 5C and D). These data indicated that the deficiency of Polβ increased the malignant proliferation of SHEE cells treated with NMBA.

Table 1

Comparison of exome sequencing results with mutations reported in the COSMIC database and GEPIA database.

Gene	GeneName	REF	ALT	Func	ExonicFunc	AAChange	Expression in Esophageal cancer	FATHMM prediction
NM_001205282	Gm14496	C	A	exonic	missense SNV	Gm14496:NM_001205282:exon4:c.C1462A:p.Q488K		
NM_182694	Ggn	G	A	exonic	missense SNV	Ggn:NM_182694:exon2:c.G418A:p.G140R		
NM_001303502; NM_007455	Ap1g2	C	T	exonic	missense SNV	Ap1g2:NM_001303502:exon19:c.G1462A: p.V488I,Ap1g2:NM_007455:exon20: c.G2104A:p.V702I		Pathogenic
NM_175501	Adamts12	G	T	exonic	missense SNV	Adamts12:NM_175501:exon23:c.G4553T:p.C1518F	over(*)	Pathogenic
NM_001198565; NM_001198566; NM_172294	Sulf1	G	A	exonic	missense SNV	Sulf1:NM_001198566:exon14:c.G1918A: p.D640N,Sulf1:NM_001198565:exon15: c.G1918A:p.D640N,Sulf1: NM_172294:exon15:c.G1918A:p.D640N	over(*)	Pathogenic
NM_026187	Ankzf1,Glb1l	T	G	exonic	missense SNV	Ankzf1:NM_026187:exon11:c.T2012G:p.F671C		Pathogenic
NM_013701;NM_145079; NM_201410;NM_201641; NM_201642;NM_201643; NM_201644;NM_201645	Ugt1a1,Ugt1a5, Ugt1a6b,Ugt1a9, Ugt1a7c,Ugt1a10, Ugt1a6a, Ugt1a2	C	T	exonic	missense SNV	Ugt1a2:NM_013701:exon5:c.C1427T: p.P476L,Ugt1a10:NM_201641:exon5: c.C1418T:p.P473L,Ugt1a7c:NM_201642:exon5: c.C1421T:p.P474L,Ugt1a5:NM_201643: exon5:c.C1415T:p.P472L,Ugt1a9: NM_201644:exon5:c.C1412T: p.P471L,Ugt1a1:NM_201645:exon5: c.C1433T:p.P478L,Ugt1a6a: NM_145079:exon6: c.C1421T:p.P474L,Ugt1a6b: NM_201410:exon6:c.C1421T:p.P474L		Pathogenic
NM_153108	Defb8	T	C	exonic	missense SNV	Defb8:NM_153108:exon2:c.A92G:p.Y31C		
NM_153108	Defb8	G	C	exonic	missense SNV	Defb8:NM_153108:exon2:c.C89G:p.T30S		
NM_153108	Defb8	A	T	exonic	missense SNV	Defb8:NM_153108:exon2:c.T81A:p.D27E		
NM_207658	Defa22	A	G	exonic	missense SNV	Defa22:NM_207658:exon1:c.A31G:p.I11V		
NM_001079933	Defa26	G	A	exonic	missense SNV	Defa26:NM_001079933:exon1:c.G124A:p.V42M		
NM_175833	Cdv3	T	G	exonic	missense SNV	Cdv3:NM_175833:exon5:c.A793C:p.N265H		
NM_011686	Vmn2r88	C	A	exonic	missense SNV	Vmn2r88:NM_011686:exon2:c.C422A:p.A141D		
NM_001102584	Vmn2r114	G	C	exonic	missense SNV	Vmn2r114:NM_001102584:exon6:c.C1860G:p.N620K		
NM_025383	Necap2	C	T	exonic	missense SNV	Necap2:NM_025383:exon2:c.G140A:p.R47Q		Pathogenic
NM_172298	Tshz3	C	T	exonic	missense SNV	Tshz3:NM_172298:exon2:c.C3203T:p.P1068L		Pathogenic

(*continued on next page*)

Table 1 (*continued*)

Gene	GeneName	REF	ALT	Func	ExonicFunc	AAChange	Expression in Esophageal cancer	FATHMM prediction
NM_030207	Sfi1	T	C	exonic	missense SNV	Sfi1:NM_030207:exon29:c.A3197G:p.H1066R		
NM_144848	Eppk1	T	A	exonic	missense SNV	Eppk1:NM_144848:exon2:c.A9143T:p.E3048V	over(*)	Pathogenic
NM_080457	Muc4	A	G	exonic	missense SNV	Muc4:NM_080457:exon4:c.A5011G:p.S1671G		
NM_080457	Muc4	T	G	exonic	missense SNV	Muc4:NM_080457:exon4:c.T5013G:p.S1671R		
NM_001100616	Vmn2r121	G	A	exonic	missense SNV	Vmn2r121:NM_001100616:exon3:c.C1199T:p.A400V		
NM_030194;NM_175397	Sp110	G	A	exonic	missense SNV	Sp110:NM_030194:exon7:c.C754T:p.R252C, Sp110:NM_175397:exon8:c.C754T:p.R252C	over(*)	
NM_001281466	Mroh2a	G	A	exonic	missense SNV	Mroh2a:NM_001281466:exon29:c.G3074A:p.S1025N		
NM_001281516	Gm21671	T	A	exonic	missense SNV	Gm21671:NM_001281516:exon1:c.A51T:p.E17D		
NM_001177579	Gm10471	A	T	exonic	missense SNV	Gm10471:NM_001177579:exon3:c.T320A:p.F107Y		
NM_001170884	Trim43b	T	A	exonic	missense SNV	Trim43b:NM_001170884:exon3:c.A479T:p.K160I		
NM_030207	Sfi1	A	G	exonic	missense SNV	Sfi1:NM_030207:exon12:c.T1094C:p.F365S		
NM_080457	Muc4	C	T	exonic	missense SNV	Muc4:NM_080457:exon3:c.C3694T:p.L1232F		
NM_080457	Muc4	A	T	exonic	missense SNV	Muc4:NM_080457:exon4:c.A4298T:p.N1433I		
NM_013630	Pkd1	G	A	exonic	missense SNV	Pkd1:NM_013630:exon45:c.G12374A:p.R4125H		Pathogenic
NM_001281466	Mroh2a	C	T	exonic	missense SNV	Mroh2a:NM_001281466:exon6:c.C605T:p.T202M		
NM_021559	Zfp24	T	C	exonic	missense SNV	Zfp24:NM_021559:exon2:c.A31G:p.I11V		
NM_011794		A	T	exonic	missense SNV	Bpnt1:NM_011794:exon7:c.A571T:p.I191F		

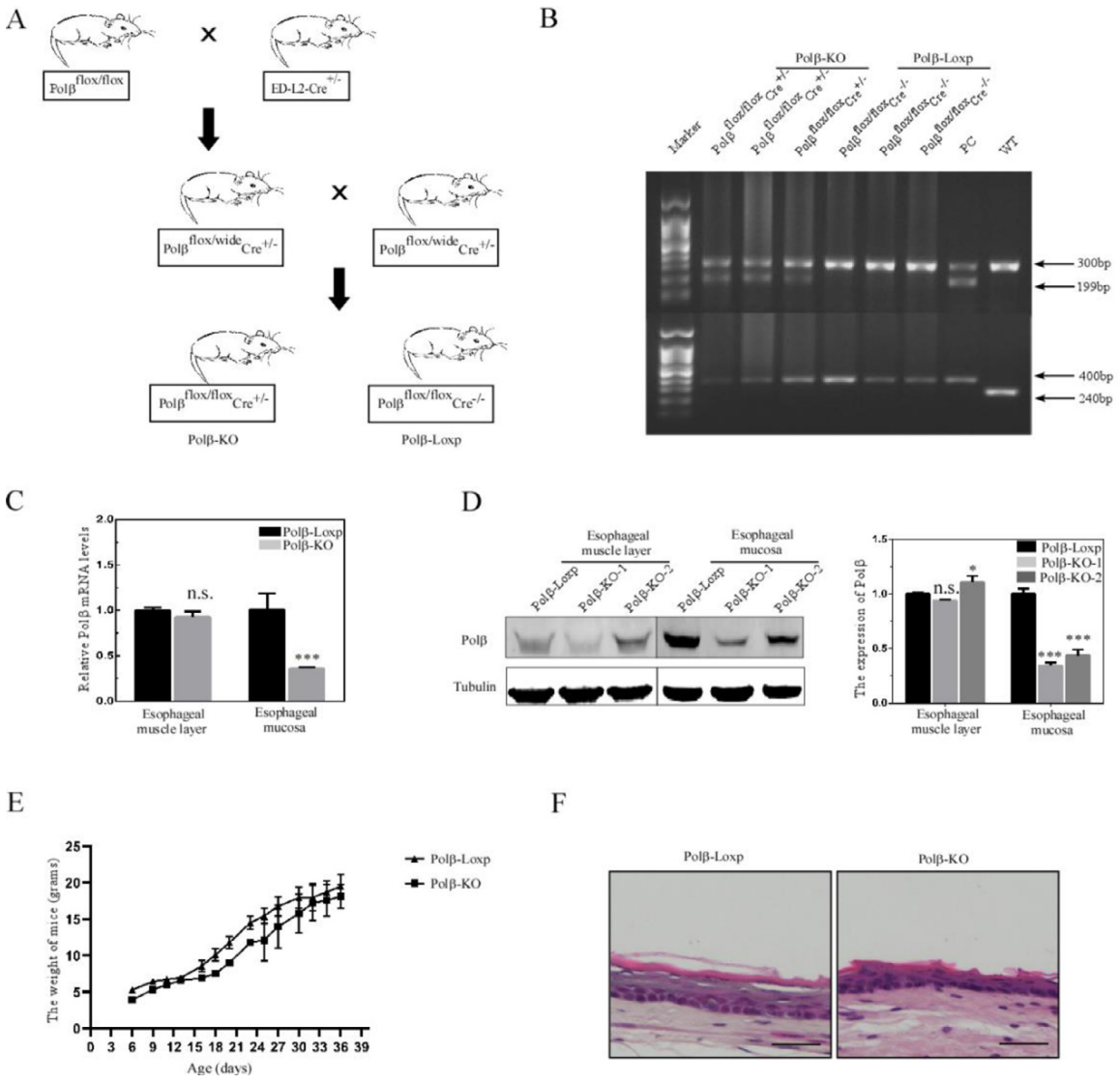

Fig. 1. Establishment of Polβ knockout mice model. (A) The flocwchart of Pol$\beta^{flox/flox}$ mice crossed with ED-L2-CRE+/- mice to create conditional knockout mice with specific ablation of Polβ in esophageal epithelial cells. (B) The genotype of mice was detected through PCR. (folxed Polβ:400bp, wild:240bp, ED-L2-CRE$^{+/-}$: 199bp, PC = positive control, WT = wild type control) (C) The mRNA levels of Polβ in esophageal muscle layer and esophageal mucosa of Polβ-Loxp and Polβ-KO mice was detected by qPCR. (D) The protein expression of Polβ in esophageal muscle layer and esophageal mucosa of Polβ-Loxp and Polβ-KO mice was detected by western blotting. (E) The weight curves of Polβ-Loxp and Polβ-KO mice after they were birth 6 d. (F) The esophagus tissue structure of Polβ-Loxp and Polβ-KO mice was examined by HE staining when they were 36 d old. (*, $P < 0.05$; ***, $P < 0.001$)

Knockdown of Polβ promotes expression of PLK1 and cell cycle progression after NMBA treatment

Gene expression profile analysis showed that the expression of PLK1 increased in Polβ-KO mice. PLK1 is a serine or threonine kinase protein that can regulate the normal operation of the cell cycle. PLK1 is highly expressed in a variety of cancers, and inhibition of PLK1 can effectively inhibit the proliferation of cancer cells [20, 21]. In addition, it is interesting that PLK1 can enhance the adaptability of cells to DNA damage, so that cells continue to divide and proliferate, and eventually accumulate damage and become malignant tumors [22]. In this study, we found that the mRNA level of PLK1 in Polβ-KO mice was increased, which is consistent with the gene expression profile results (Fig. 4F). Phosphorylation of T210 site facilitated the activity of PLK1, we found that the expression of PLK1^{T210} was increased in the Polβ-KO mice compared to the Polβ-Loxp mice through immunohistochemistry (Fig. 4G). In addition, we also confirmed that the deficiency of Polβ enhanced the expression of PLK1 and PLK1^{T210} after NMBA treatment, and promoted the cells transformation to into G2/M phase (Fig. 6).

Discussion

Esophageal cancer is one of the most common fatal cancers, the incidence and mortality of which rank seventh and sixth worldwide, respectively. The incidence rate of esophageal cancer is very high in China with male patients comprising the majority [23]. Early diagnosis and treatment are effective measures to improve the survival rate and prognosis of esophageal cancer patients [24, 25]. Effective measures at the stage of esophageal precancerous lesions can stop its development into cancer [26]. It is important to clarify the pathogenesis of esophageal precancerous lesions, which may facilitate active prevention and finding effective treatments to improve the survival rate

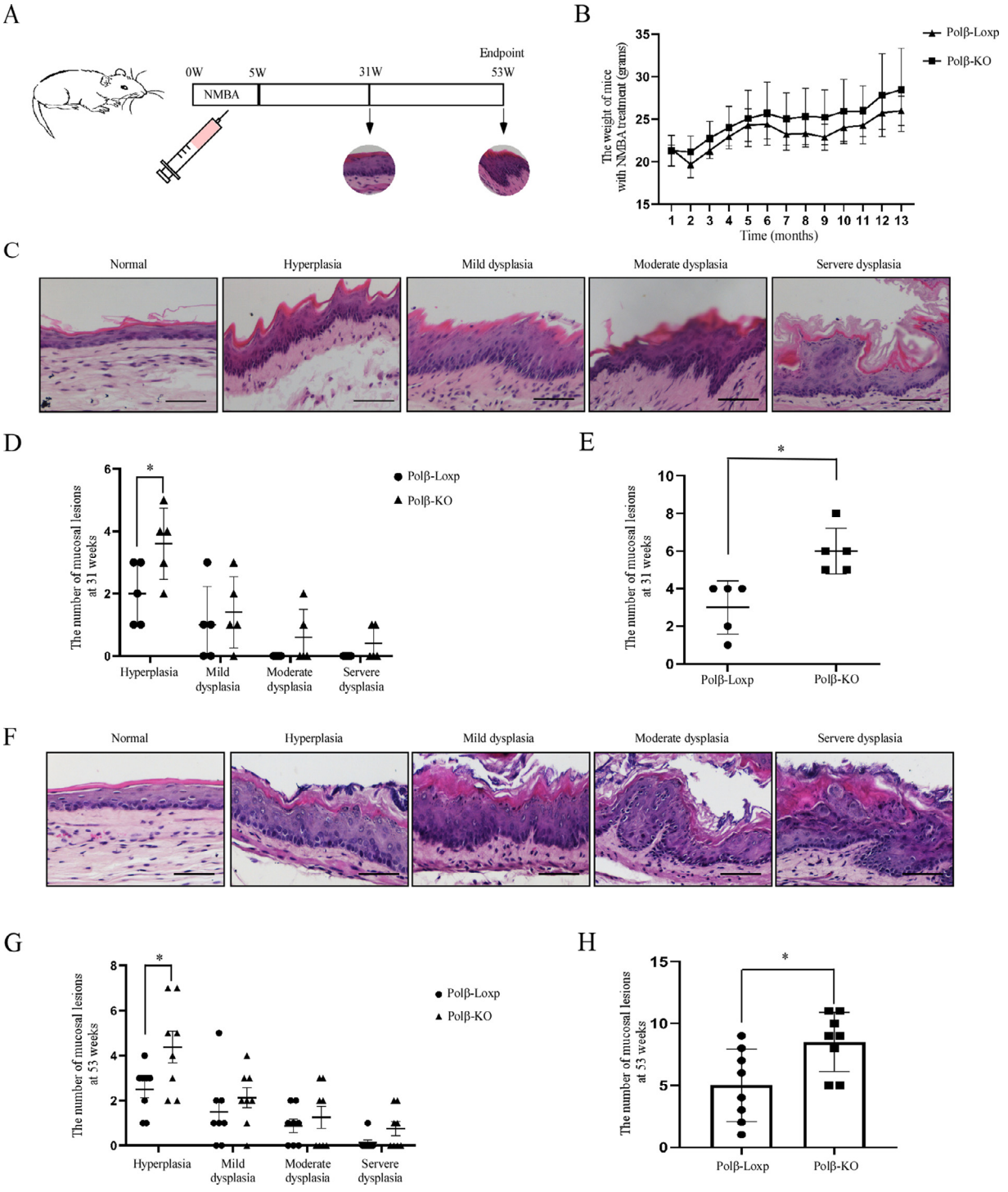

Fig. 2. The deletion of Polβ promoted the occurrence of esophageal precancerous lesions after NMBA induction. (A) The time diagram of animal experiment. 0 to 5w, the period of NMBA(2mg/kg) induction; at 31 and 53 wk, the mice were euthanized and detected the esophageal lesions by HE staining. (B) The growth curves of Polβ-Loxp and Polβ-KO mice after NMBA induction. (C) Representative pictures for different dysplasia of esophageal precancerous lesions were shown at 31 wk. (D and E) The classification and number of esophageal mucosa lesions in Polβ-Loxp and Polβ-KO mice at 31 wk were counted. (F) Representative pictures for different dysplasia of esophageal precancerous lesions were shown at 53 wk. (G and H) The classification and number of esophageal mucosa lesions in Polβ-Loxp and Polβ-KO mice at 53 wk were counted. (*, $P < 0.05$)

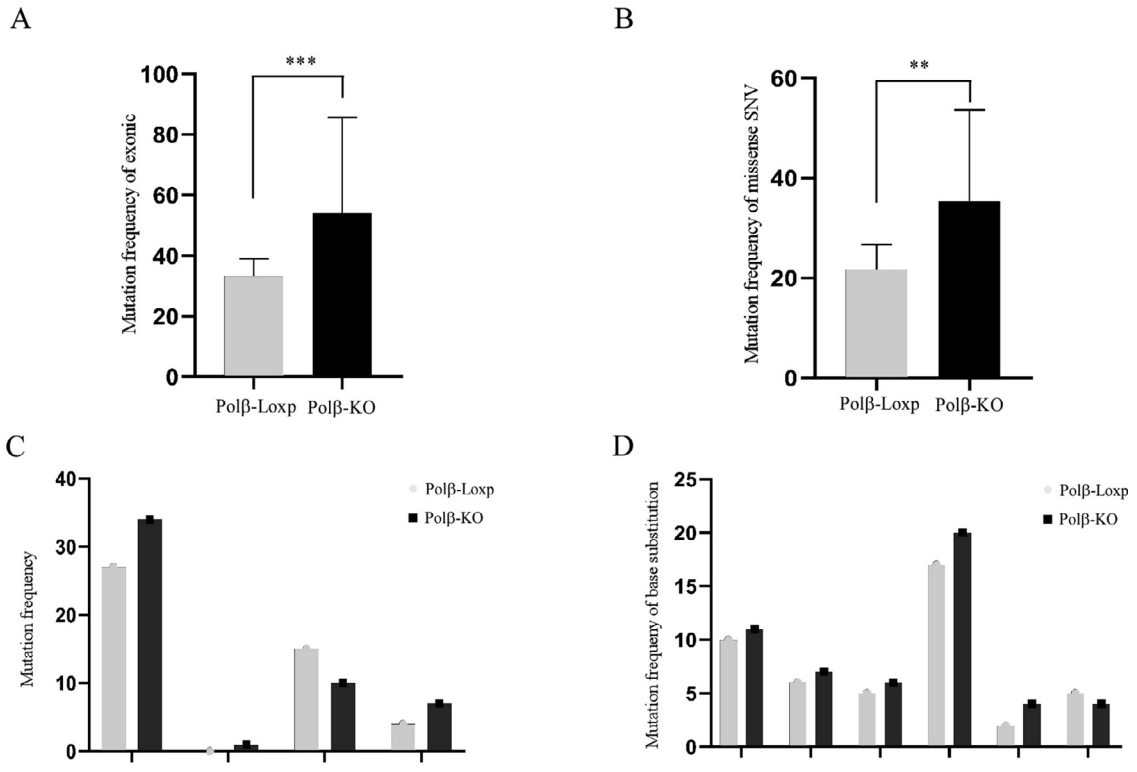

Fig. 3. Whole exome sequencing showed that gene mutations of Polβ-Loxp and Polβ-KO mice. (A) The picture showed that frequency of different mutation types in genes exon region of Polβ-Loxp and Polβ-KO mice. (B) The graph displayed the mutation frequency of base substitution in Polβ-Loxp and Polβ-KO mice.

and prognosis of cancer patients. N-nitrosomethylbenzylamine (NMBA) is a metabolite of nitrosamine compounds and a known chemical carcinogen. In the esophageal epithelium of humans and rats, nitrosamine compounds are converted into metabolites that alkylate DNA at the N and O^6 positions of guanine. The persistence, accumulation, and DNA mutation ability of O^6-methylguanine are thought to play major roles in carcinogenesis [27]. The appropriate dose and route of administration of NMBA are effectively used to establish esophageal cancer models in rats. Similarly, NMBA also induces dysplasia of the esophageal mucosa in mice [15]. In this study, NMBA as a DNA-damaging agent was used to induce esophageal precancerous lesions to explore the relationship between Polβ deletion and esophageal precancerous lesions.

Genomic stability plays an important role in maintaining normal growth and development of the body. A series of reports have suggested that genomic instability is associated with neurodegenerative diseases, aging, immunodeficiency, and even carcinogenesis [28-30]. DNA damage caused by various endogenous or exogenous factors and inactivation or abnormality of the DNA damage repair pathway are major causes of genomic instability. In the base excision repair process, the normal Polβ function is critical to repair damaged DNA and maintain genomic stability [31]. A lack of Polβ increases the sensitivity of mouse embryonic fibroblasts (MEFs) to the DNA damage inducer methyl methanesulfonate (MMS) and other DNA polymerases cannot compensate for Polβ under the action of high concentrations of MMS. Additionally, chromosome destruction in MEFs that lack Polβ increases compared with wildtype cells after treatment with alkylating agents [4, 32-34]. Abnormal expression and mutation of Polβ are related to the occurrence of esophageal cancer, but the relationship of Polβ deletion with esophageal cancer is unclear. In this study, we established Polβ knockout mice to explore the role of Polβ deficiency in esophageal precancerous lesions. The results showed that deletion of Polβ led to severe dysplasia of esophageal mucosa and the number of lesions at various levels increased in Polβ-KO mice at 2 mg/kg NMBA treatment by subcutaneous injection compared with Polβ-Loxp mice (Fig. 2). Additionally, whole exon sequencing showed that deletion of Polβ increased the frequency of gene mutations (Fig. 3). We speculated that DNA damage cannot be repaired because of the loss of Polβ and leads to accumulation of mutations in the genome. We found nine known driver genes related to esophageal cancer in accordance with the COSMIC database, among which adams12, sulf1, eppk1, and sp110 are highly expressed in esophageal cancer in accordance with the GEPIA database (Table 1 and Supplementary Fig 2). These results indicate that Polβ deficiency promotes the occurrence of esophageal precancerous lesions, which may be partly due to increased gene mutation frequency.

Polβ plays a pivotal role in controlling the balance of cell proliferation. Overexpression of the PolβT889C mutant promotes the proliferation and invasion of gastric cancer cells [35]. Natamycin inhibits the proliferation of prostate cancer cells by blocking Polβ [36], which indicates that abnormal Polβ expression is closely related to cell proliferation. Ki67 is mainly expressed in proliferating cell, and p-Ki67 has been identified as a diagnostic marker and prognostic indicator of various tumors. The ERK signaling pathway regulates cell proliferation and differentiation, and its abnormal activation promotes the occurrence of cancer. Similarly, the PI3K/AKT cascade coordinates insulin signal transduction and mediates cell proliferation and survival during growth [37-39]. In this study, we found that the degree of esophageal precancerous lesions in Polβ-KO mice was more serious than that in Polβ-Loxp mice, and the phosphorylation levels of proliferation-associated proteins Ki67, AKT, PI3K, and ERK were increased in the esophageal mucosa of Polβ-KO mice. The insulin signaling pathway induces phosphorylation events and activates the PI3K signaling cascade [40]. Additionally, we found that knockdown of Polβ enhanced the proliferation and colony-forming ability of SHEE cells. These results suggest that loss of Polβ promotes the

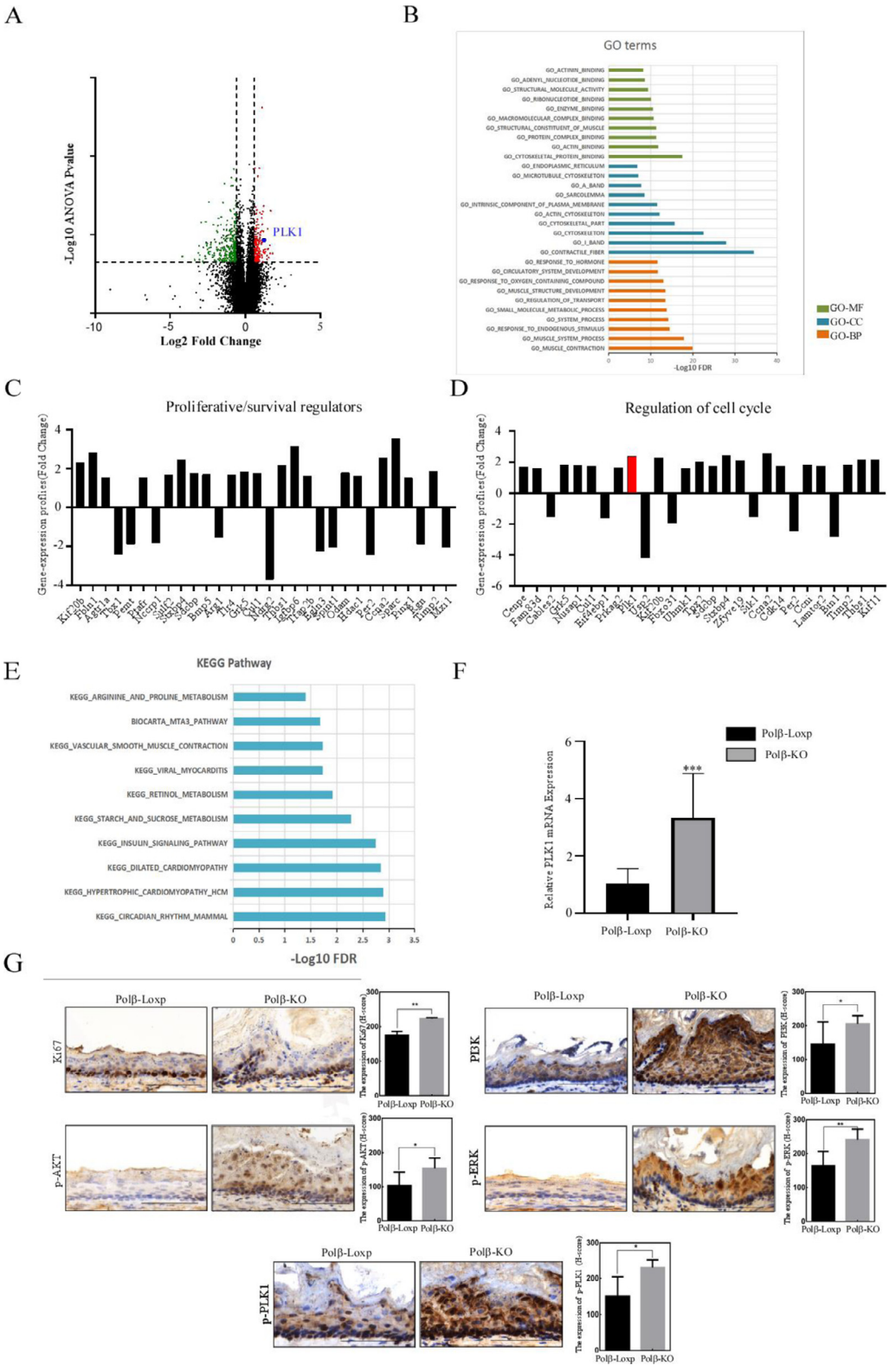

Fig. 4. Gene expression profile analysis revealed the mechanism of Polβ deficiency promoted esophageal precancerous lesions after NMBA treatment. (A) Volcano plot showed that 504 gene changed significantly (*|fold change|>1.5 and P-value < 0.05*). Green dots represent down-regulated gene, and red dots represent upregulated gene. (B) The histogram showed GO annotation, and enrichment analysis of genes with significant changes after Polβ deletion induced by NMBA was performed. The FDR (-log10) of each GO term was shown. (C and D) The graph showed the genes with significant changes related to cell proliferation, survival and cell cycle. (E) The bar chart showed that KEGG enrichment analysis was performed for genes with significant changes in the Polβ-KO and Polβ-Loxp mice treated by NMBA according to the FDR (-log10). (F) The relative mRNA expression of PLK1 in esophageal mucosa of Polβ-KO and Polβ-Loxp mice. (G) Immunohistochemistry showed the expression of Ki67, PI3K, p-AKT, p-ERK and p-PLK1 in esophageal mucosa of Polβ-Loxp and Polβ-KO mice. (*, $P < 0.05$; ** $P < 0.01$)

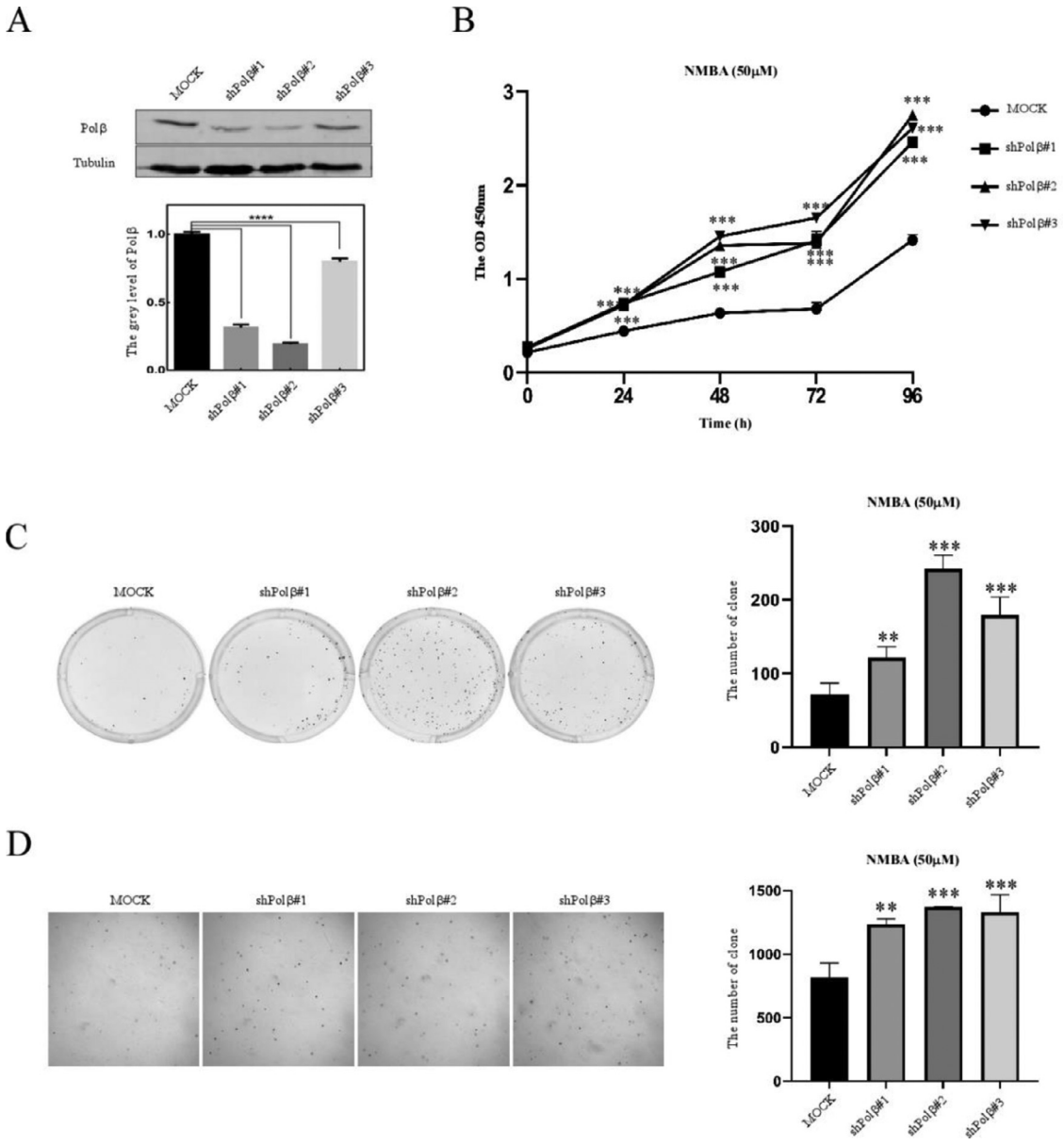

Fig. 5. The loss of Polβ promoted proliferation of esophageal epithelial cells (SHEE) after NMBA induction. (A) The Polβ knockdown effect was detected by western blotting in SHEE cell. (B) The OD value of cells were detected at 450 nm by a microplate reader at different time after NMBA continuous induction 10 d. (C and D) The size and number of SHEE cells clone were detected by clone formation assay and anchorage-independent cell growth assay after NMBA continuous induction 10 d. (** $P < 0.01$;*** $P < 0.000$)

occurrence of esophageal precancerous lesions accompanied by activation of cell proliferation and cell cycle-related pathways.

PLK1 is a serine/threonine kinase involved in the regulation of cell cycle progression including mitotic entry, spindle formation, and cytokinesis. Phosphorylation of the T210 site increases the activity of PLK1 [41]. Mounting evidence suggests that PLK1 is overexpressed in various tumors and related to the cancer stage, a poor prognosis, and survival rate, which is considered to be an oncogene [42, 43]. It is recognized that PLK1 overexpression causes tumorigenesis by activating the PI3K signaling pathway [44]. Additionally, PLK1 allows cells with damaged DNA to continue to divide and proliferate by enhancing the adaptability of checkpoints after human osteosarcoma cells are damaged by ionizing radiation, and then the risk of genomic instability leading to cancer increases [22]. In our study, the expression of PLK1 and PLK1^{T210} in Polβ-KO mice and SHEE cells with Polβ deficiency was increased after NMBA treatment and the number of cells was increased in G2/M phase. Combined with the frequency of genome mutations increased in Polβ-KO mice, we suspect that increasing PLK1 under loss of Polβ plays a major role in the esophagus precancerous lesions. High expression of PLK1 may promote the damage adaptability of cells, which ultimately increases genomic instability and promotes the occurrence of esophageal precancerous lesions. However, the specific mechanism of the increase in PLK1 expression caused by Polβ remains unclear and requires further exploration.

In conclusion, our study indicates that deficiency of Polβ increases the frequency of gene mutations and promotes the occurrence of esophageal precancerous lesions of mice as well as the proliferation and colony formation ability of SHEE cells. These findings suggest that maintaining the balance of Polβ plays an important role in preventing esophageal precancerous lesions.

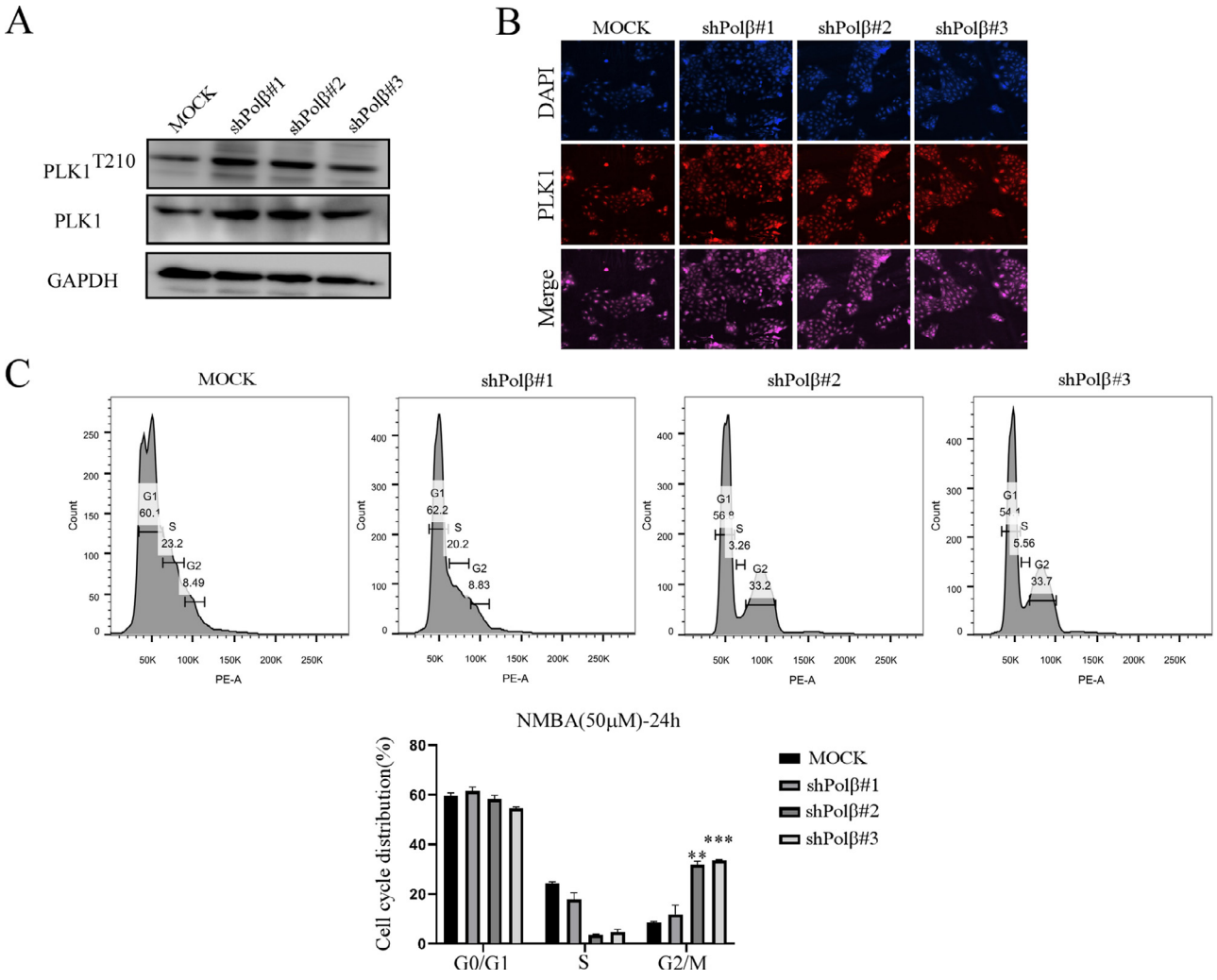

Fig. 6. The deficiency of Polβ promotes the expression of PLK1 and cell cycle progress after NMBA induced. (A) The expression level of PLK1 and PLK1^{T210} was detected by western blotting after SHEE cells with Polβ knockdown were treated with NMBA. (B) The fuorescence intensity of PLK1 in the nucleus was detected by immunofluorescence assay after SHEE cells with Polβ knockdown were treated with NMBA. (C) The progression of cell cycle was detected by a FACScan flow cytometry after SHEE cells with Polβ knockdown was treated with NMBA. (** $P < 0.01$; *** $P < 0.001$)

Declaration of competing interest

The authors have no conflict of interest.

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Author contributions

ZJM, CXH, ZYY, QJC, and DYW designed the study. ZYY, QJC, DYW, ZLL and NTT performed the experiments. ZJM, ZYY, QJC and DYW analyzed data and wrote the manuscript. YBY, QY, LJ, LKD, DZM, JG and CXH reviewed and edited the manuscript. All authors read and approved the final manuscript.

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References

[1] Chang LM, Bollum FJ. Low molecular weight deoxyribonucleic acid polymerase in mammalian cells. *J Biol Chem* 1971;**246**:5835–7.

[2] Krokan HE, Bjoras M. Base excision repair. *Cold Spring Harb Perspect Biol* 2013;**5**:a12583.

[3] Prasad R, Beard WA, Chyan JY, Maciejewski MW, Mullen GP, Wilson SH. Functional analysis of the amino-terminal 8-kDa domain of DNA polymerase beta as revealed by site-directed mutagenesis. DNA binding and 5′-deoxyribose phosphate lyase activities. *J BIOL CHEM* 1998;**273**:11121–6.

[4] Sobol RW, Prasad R, Evenski A, Baker A, Yang XP, Horton JK, Wilson SH. The lyase activity of the DNA repair protein beta-polymerase protects from DNA-damage-induced cytotoxicity. *NATURE* 2000;**405**:807–10.

[5] Illuzzi JL, Wilson DR. Base excision repair: contribution to tumorigenesis and target in anticancer treatment paradigms. *CURR MED CHEM* 2012;**19**:3922–36.

[6] Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, Prasad R, Rajewsky K, Wilson SH. Requirement of mammalian DNA polymerase-beta in base-excision repair. *NATURE* 1996;**379**:183–6.

[7] Sobol RW, Wilson SH. Mammalian DNA beta-polymerase in base excision repair of alkylation damage. *Prog Nucleic Acid Res Mol Biol* 2001;**68**:57–74.

[8] Kim J, Kim J, Lee Y. DNA polymerase beta deficiency in the p53 null cerebellum leads to medulloblastoma formation. *Biochem Biophys Res Commun* 2018;**505**:548–53.

[9] Guo Z, Zheng L, Dai H, Zhou M, Xu H, Shen B. Human DNA polymerase beta polymorphism, Arg137Gln, impairs its polymerase activity and interaction with PCNA and the cellular base excision repair capacity. *NUCLEIC ACIDS RES* 2009;**37**:3431–41.

[10] Yamtich J, Nemec AA, Keh A, Sweasy JB. A germline polymorphism of DNA polymerase beta induces genomic instability and cellular transformation. *PLOS GENET* 2012;**8**:e1003052.

[11] Zhou T, Pan F, Cao Y, Han Y, Zhao J, Sun H, Zhou X, Wu X, He L, Hu Z, et al. R152C DNA Pol beta mutation impairs base excision repair and induces cellular transformation. *Oncotarget* 2016;**7**:6902–15.

[12] Dong Z, Zhao G, Zhao Q, Yang H, Xue L, Tan X, Zheng N. [A study of DNA polymerase beta mutation in human esophageal cancer]. *Zhonghua Yi Xue Za Zhi* 2002;**82**:899–902.

[13] Yang H, Jia X, Chen X, Yang CS, Li N. Time-selective chemoprevention of vitamin E and selenium on esophageal carcinogenesis in rats: the possible role of nuclear factor kappaB signaling pathway. *INT J CANCER* 2012;**131**:1517–27.

[14] Maliakal P, Abdelrahim M, Sankpal UT, Maliakal C, Baker CH, Safe S, Herrera LJ, Abudayyeh A, Kaja S, Basha R. Chemopreventive effects of tolfenamic acid against esophageal tumorigenesis in rats. *Invest New Drugs* 2012;**30**:853–61.

[15] Jenkins TD, Mueller A, Odze R, Shahsafaei A, Zukerberg LR, Kent R, Stoner GD, Rustgi AK. Cyclin D1 overexpression combined with N-nitrosomethylbenzylamine increases dysplasia and cellular proliferation in murine esophageal squamous epithelium. *ONCOGENE* 1999;**18**:59–66.

[16] Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, Gupta A, Blackwood M, Stoner GD. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries. *CANCER RES* 2001;**61**:6112–19.

[17] Wu Y, Zhao J, Dong S, Wang Y, Li A, Jiang Y, Chen Z, Li C, Wang W, Zhang Z. Whole-exome and RNA sequencing reveal novel insights into the pathogenesis of HPV associated cervical cancer. *CANCER BIOMARK* 2019;**25**:341–50.

[18] Lian Z, Lee EK, Bass AJ, Wong KK, Klein-Szanto AJ, Rustgi AK, Diehl JA. FBXO4 loss facilitates carcinogen induced papilloma development in mice. *CANCER BIOL THER* 2015;**16**:750–5.

[19] Masaoka A, Horton JK, Beard WA, Wilson SH. DNA polymerase beta and PARP activities in base excision repair in living cells. *DNA Repair (Amst)* 2009;**8**:1290–9.

[20] Donizy P, Halon A, Surowiak P, Kaczorowski M, Kozyra C, Matkowski R. Augmented expression of Polo-like kinase 1 is a strong predictor of shorter cancer-specific overall survival in early stage breast cancer at 15-year follow-up. *ONCOL LETT* 2016;**12**:1667–74.

[21] Liu X, Choy E, Harmon D, Yang S, Yang C, Mankin H, Hornicek FJ, Duan Z. Inhibition of polo-like kinase 1 leads to the suppression of osteosarcoma cell growth in vitro and in vivo. *Anticancer Drugs* 2011;**22**:444–53.

[22] Syljuasen RG, Jensen S, Bartek J, Lukas J. Adaptation to the ionizing radiation-induced G2 checkpoint occurs in human cells and depends on checkpoint kinase 1 and Polo-like kinase 1 kinases. *CANCER RES* 2006;**66**:10253–7.

[23] Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 2021;**71**(3):209–49.

[24] Naveed M, Kubiliun N. Endoscopic treatment of early-stage esophageal cancer. *CURR ONCOL REP* 2018;**20**:71.

[25] May A, Ell C. Diagnosis and treatment of early esophageal cancer. *Curr Opin Gastroenterol* 2006;**22**:433–6.

[26] Vo T, Jang WJ, Jeong CH. Leukotriene A4 hydrolase: an emerging target of natural products for cancer chemoprevention and chemotherapy. *Ann N Y Acad Sci* 2018;**1431**:3–13.

[27] Siglin JC, Morse MA, Schut HA, Geil RG, Conran PB, Stoner GD. O6-methylguanine levels and histopathological changes in the rat esophagus and liver following single and repeated administration of N-nitrosomethylbenzylamine. *CARCINOGENESIS* 1996;**17**:1135–40.

[28] Vijg J, Dong X, Milholland B, Zhang L. Genome instability: a conserved mechanism of ageing? *ESSAYS BIOCHEM* 2017;**61**:305–15.

[29] Wang J, Lindahl T. Maintenance of Genome Stability. *Genomics Proteomics Bioinformatics* 2016;**14**:119–21.

[30] Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. *NAT REV CANCER* 2016;**16**:35–42.

[31] Sobol RW. Genome instability caused by a germline mutation in the human DNA repair gene POLB. *PLOS GENET* 2012;**8**:e1003086.

[32] Yamamoto M, Yamamoto R, Takenaka S, Matsuyama S, Kubo K. Abundance of BER-related proteins depends on cell proliferation status and the presence of DNA polymerase beta. *J RADIAT RES* 2015;**56**:607–14.

[33] Ochs K, Sobol RW, Wilson SH, Kaina B. Cells deficient in DNA polymerase beta are hypersensitive to alkylating agent-induced apoptosis and chromosomal breakage. *CANCER RES* 1999;**59**:1544–51.

[34] Yamamoto R, Umetsu M, Yamamoto M, Matsuyama S, Takenaka S, Ide H, Kubo K. AP endonuclease knockdown enhances methyl methanesulfonate hypersensitivity of DNA polymerase beta knockout mouse embryonic fibroblasts. *J RADIAT RES* 2015;**56**:462–6.

[35] Tan X, Wang H, Luo G, Ren S, Li W, Cui J, Gill HS, Fu SW, Lu Y. Clinical significance of a point mutation in DNA polymerase beta (POLB) gene in gastric cancer. *INT J BIOL SCI* 2015;**11**:144–55.

[36] Vasquez JL, Lai Y, Annamalai T, Jiang Z, Zhang M, Lei R, Zhang Z, Liu Y, Tse-Dinh YC, Agoulnik IU. Inhibition of base excision repair by natamycin suppresses prostate cancer cell proliferation. *BIOCHIMIE* 2020;**168**:241–50.

[37] Li LT, Jiang G, Chen Q, Zheng JN. Ki67 is a promising molecular target in the diagnosis of cancer (review). *MOL MED REP* 2015;**11**:1566–72.

[38] Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis. *EXP THER MED* 2020;**19**:1997–2007.

[39] Lien EC, Dibble CC, Toker A. PI3K signaling in cancer: beyond AKT. *CURR OPIN CELL BIOL* 2017;**45**:62–71.

[40] Hopkins BD, Goncalves MD, Cantley LC. Insulin-PI3K signalling: an evolutionarily insulated metabolic driver of cancer. *NAT REV ENDOCRINOL* 2020;**16**:276–83.

[41] Liu Z, Sun Q, Wang X. PLK1, a potential target for cancer therapy. *TRANSL ONCOL* 2017;**10**:22–32.

[42] Lin P, Wen DY, Dang YW, He Y, Yang H, Chen G. Comprehensive and integrative analysis reveals the diagnostic, clinicopathological and prognostic significance of polo-like kinase 1 in hepatocellular carcinoma. *CELL PHYSIOL BIOCHEM* 2018;**47**:925–47.

[43] Weichert W, Denkert C, Schmidt M, Gekeler V, Wolf G, Kobel M, Dietel M, Hauptmann S. Polo-like kinase isoform expression is a prognostic factor in ovarian carcinoma. *Br J Cancer* 2004;**90**:815–21.

[44] de Carcer G. The mitotic cancer target polo-like kinase 1: oncogene or tumor suppressor? *Genes (Basel)* 2019:10.