·Esophageal Cancer Column·

Differential gene expression profiles of DNA repair genes in esophageal cancer cells after X-ray irradiation

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[Abstract] Background and Objective: Various factors affect the radioresistance of tumor cells, with unknown molecular mechanism(s). Many genes have been found to associate with the radioresistance of tumor cells, however, the precise mechanism of these genes have not been elucidated. This paper was to analyze the differential expressions of DNA repair genes in esophageal carcinoma cells at different time after X-ray irradiation, and to investigate the role of these DNA repair genes in radiation resistance. **Methods:** Esophageal cancer parental cells Seg-1 were treated with continuous 2 Gy of fractionated irradiation until the total dose reached 60 Gy to establish the radioresistant cell line Seg-1R. Total RNA was extracted from each cell line at 0, 8, and 24 h after irradiation. Illumine Human-6 V3 microarray was used to identify differentially expressed genes between parental and radioresistant cells. Ten genes involved in DNA repair were obtained and their expressions at different time points after irradiation were analyzed by Gene Ontology analysis. **Results:** Ten DNA repair associated genes were found to be differentially expressed. Three of these genes, SLK, HMGB1, and PMS1, were not only differentially expressed between parental and radioresistant cell lines, but also expressed differently at different time points after irradiation in the same cell line. **Conclusions:** PMS1 may be an important factor involved in the mechanism of radioresistance of esophageal carcinoma cells.

Key words: Esophageal carcinoma, cDNA microarray, radiosensitive, PMS1

Esophageal cancer is a global distributed malignant cancer. China is one of countries with high incidence of esophageal cancer[1]. Radiotherapy has been confirmed as one of the preferential treatment of esophageal cancer as described in National Comprehensive Cancer Network (NCCN) (2009 version). In recent years, the survival rate of patients with esophageal cancer undergoing radiotherapy has greatly improved, with the similar rate to those treated with surgery (26%–32%). However, local recurrence is still a treatment failure[2,3]. Low rate of local control, commonly caused by radioresistance, is the bottleneck of radiotherapy.

Many factors contribute to tumor resistance in clinical radiotherapy, including intrinsic radiosensitivity of tumor cells, percentage of hypoxic cells, radiation damage repair, and clinical status. The complex molecular mechanism is the most important factor of radioresistance. Many genes have been found to associate with tumor radioresistance, however, the precise mechanism was still unknown. Accumulating evidence show that DNA is the key target for radiation treatment inducing various radiation effects, including cell death, gene mutation, and tumorigenesis[4]. Both of single-stranded and double-stranded DNA breaks occur after X-ray irradiation. Double-stranded breaks are considered to be the most serious damage to chromosome caused by irradiation. Two processes consisting of homologous and non-homologous recombination repair DNA damage[5].

DNA damage repair increases the radioresistance of

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cancer cells. Dys-regulation of DNA dam age repair genes plays critical roles in this process. All of the previous studies on gene expression profiles of radioresistance can be divided into two types: one part of studies focus on analyzing the function of single gene[6-8], and the other studies, however, only examine the differentially expressed genes by microarray in cancer cells without irradiation, showing no change of differential gene expression after radiation treatment[9,10].

Microarray has been known as a powerful tool for gene function analysis on mRNA level, with the advantages of high throughput, microminiaturization, and automation comparing with traditional methods such as cDNA library screening, Northern blot analysis, and polymerase chain reaction (PCR)[11].

Here, we established a stable radioresistant cell line, Seg-1R, through treating esophageal cancer cells Seg-1 with continuous 2 Gy of fractionated irradiation, with the total dose of 60 Gy. We further compared gene expression profiles of radioresistant cells and their corresponding parental cells at different time points after irradiation using cDNA microarray. Moreover, we classified differentially expressed genes according to their function by Gene Ontology and firstly analyzed the DNA damage repair associated genes. Meanwhile, we discussed the relationship between DNA damage repair associated genes and radioresistance in order to provide new insights for understanding the molecular mechanism of radioresistance of esophageal cancer.

Materials and Methods

Cell culture

Human esophageal cancer cells lines, Seg-1 and Seg-1R, were kindly provided by Prof. Joe Y. Chang (Department of Radiology, M.D. Anderson Cancer Center). Seg-1R cell line was established through treating esophageal cancer cells Seg-1 with continuous 2 Gy of fractionated irradiation (the total dose was 60 Gy)[12]. Cells were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37℃ in humidified 5% CO_2 incubator.

X-ray irradiation

Seg-1 and Seg-1R cells were divided into three groups: before irradiation (0 h), 8 h after irradiation, and 24 h after irradiation. Cells were cultured in 75 cm^2 flask with cell number of 1×10^7. When they reached 80% confluence, the cells were treated with X-ray irradiation with a dose of 400 cGy.

Total RNA extraction and validation

Total RNA was extracted by TRIzol reagent from cells at three time points. The integrity of RNA was measured by agarose gel electrophoresis. The concentration of RNA was validated with a UV spectrophotometer at 260/280 nm. CDNA was synthesized by reverse transcription (RT) and purified by Illumina TotalPrep RNA amplification kit.

Whole human genome microarray and data analysis

Illumine Human-6 V3 microarray was from Shanghai Biochip Co., Ltd. Each microarray, which can detect six samples at the same time, consists of 48 000 transcripts, including not only 24 000 transcripts from NCBI RefSeq but also 24 000 transcripts from UniGene, RefSeq Gnomon, and Genome-Annotation RefSeq. Each microarray was scanned by Illumina scanner and the data were analyzed by BeadStudio software. We analyzed the grayscale of microarray image using a microarray image software to get the original signal value of each gene, which is the mean of signal values which equal the effective repeat point signal values minus the background signal values.

Gene Ontology (GO) analysis: GO is a bioinformatics initiative to unify the representation of genes and gene product attributes. The aims of GO project are to annotate genes and to classify genes according to their function, biological process, and cellular components.

Screening of differentially expressed genes

(1) In order to determine the different gene expression profiles in radioresistant esophageal cancer cells treated with continuous radiation, we compared the gene expression profiles at three time points between radiosensitive cells Seg-1 and radioresistant cells Seg-1R. The expression profiles of Seg-1 cells were considered as basal value. Differentially expressed genes were identified by comparing gene expression values of two cell lines at one time point.

(2) To analyze the dynamic changes of DNA damage repair associated genes after irradiation, we compared the gene expression profiles at 0, 8, and 24 h after irradiation in two cell lines. Differentially expressed genes were identified by the expression value ratios at 8 h and 0 h or 24 h and 0 h.

The ratio value of $\geqslant 1.5$ was assessed as up-regulated genes, while the ratio value of $\leqslant 0.75$ as down-regulated genes. All differentially ex pressed genes were analyzed by Gene Ontology.

RT-PCR

The first chain of cDNA was synthesized by Reverse Transcription System kit (Promega USA). β-actin (negative control, 564 bp) and PMS1 were amplified under the same condition. PCR condition was as follows: denaturalized at 95℃ for 2 min, annealed at 55℃ for 30 s, extended at 72℃ for 1 min (30 cycles), and followed by an extension at 72℃ for 10 min. The PCR product of PMS1 was 236 bp.

The primers were as follows: β-actin, 5'-CTGGGACGACATGGAGAAAA-3' and 5'-AAGGAAGGCTGGAAGAGTGC-3'; PMS1, 5'-AAACTCCTTGGATGCTGGTG-3' and 5'-ACAACAAATTGACCCCAAGG-3'. All RT-PCR reactions were repeated three times.

Repetitive verification

To further confirm our data, we verified our results in esophageal squamous cell lines Kyse170 and Kyse170R, which was established through treating Kyse170 cells with X-ray irradiation by continuous 2 Gy of irradiation (total dose was 60 Gy, and both cell lines were kindly provided by Prof. Joe Y. Chang from M.D. Anderson Cancer Center). Similarly, we compared the expression of these DNA repair associated genes between before irradiation and at 8 h and 24 h after irradiation.

Results

Total RNA extraction and validation

The total RNA from Seg-1 and Seg-1R cells treated with irradiation was measured at three time points by agarose gel electrophoresis and the results are shown in Figure 1, suggesting that the quality of RNA extracted met the standard.

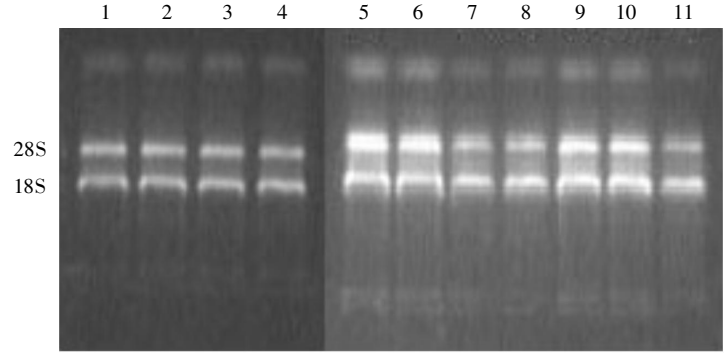

Figure 1 The result of agarose gel electrophoresis of the total RNA extracted from Seg-1 and Seg-1R cells at different time points after irradiation

Lines 1, 2, 5, 6, 9, and 10, Seg-1 cells; lines 3, 4, 7, 8, and 11, Seg-1R cells. Lines 1–4, 0 h after irradiation; lines 5–8, 8 h after irradiation; lines 9–11, 24 h after irradiation. The bands of 18S and 28S of RNA are clearly shown as a ratio of 2:1, suggesting that RNA quality met the standard.

Hybridization analysis

The data from scanning after irradiation and a summary report of Illumima microarray showed that the results of microarray (Figure 2) were reliable.

Screening of differentially expressed genes and Gene Ontology analysis

Five DNA repair associated genes (GO: 0006281) were screened (Table 1). The five genes were differentially expressed between two cell lines and the ratios of the expression of these genes in Seg-1R cells to that in Seg-1 cells increased. The ratio of SLK expression decreased to 74.8% at 0 h, but increased to 1.669 times at 24 h after irradiation. The ratio of FEN1 expression increased to 1.637 times at 24 h after irradiation. The ratio of CRY1 expression decreased at three time points. The ratio of HMGB1 expression reduced to 29.1% at 0 h, but increased to 3.708 times at 24 h after irradiation. The expression of PMS1 was up-regulated at 8 h and 24 h after irradiation (2.110 times and 6.053 times, respectively).

Ten DNA damage repair associated genes were differentially expressed in the same cell line at different time points (Table 2). Among these genes, the expression of five genes was different between radiosensitive cells and radioresistant cells. The expressions of POLB, SLK, CRY1, APEX2, and RMB14 were up-regulated at 8 h and 24 h after irradiation in Seg-1 and Seg-1R cells comparing with the expresions at 0 h. The expression profiles did not show significant changes at 8 h and 24 h after irradiation comparing with that at 0 h in Seg-1 cells, However, HMGB1 and PMS1 increased in Seg-1R cells after irradiation. The

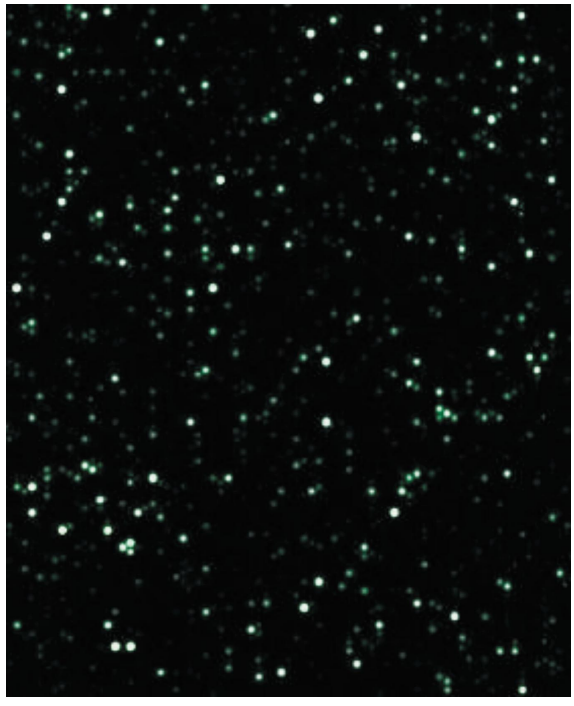

Figure 2 The gradation scanogram of chip

The gray intensity reflects fluorescent molecular strength. The molecular strength is favorable, and the homogenicity of chip is normal, suggesting that the result is reliable.

Table 1 The DNA repair genes between parental and radioresistant cells at different time points

Entrez_ Gene_ID	Symbol	Ratio		
		Seg-1R/Seg-1 0h	Seg-1R/Seg-1 8h	Seg-1R/Seg-1 24h
3146	HMGB1	**0.291**	0.984	**3.708**
5378	PMS1	1.046	**2.110**	**6.053**
9748	SLK	**0.748**	1.166	**1.669**
2237	FEN1	1.250	0.983	**1.637**
1407	CRY1	**0.093**	**0.436**	**0.241**

Note: The mean ratios of $\geqslant$ 1.5 or $\leqslant$ 0.75 are presented as boldface.

Table 2 The DNA repair genes of Seg-1 and Seg-1R cells at different time points

Entrez_ Gene_ID	Symbol	Ratio			
		Seg-1 8h/ 0h	Seg-1 24h/ 0h	Seg-1R 8h/ 0h	Seg-1R 24h/ 0h
5423	POLB	**0.725**	0.847	**0.732**	1.204
27301	APEX2	1.341	0.761	0.993	**0.706**
10432	RBM14	**2.267**	0.762	**2.058**	**0.564**
3146	HMGB1	**0.284**	**0.279**	0.961	**3.557**
5378	PMS1	1.022	1.004	**2.061**	**5.806**
9748	SLK	**0.676**	**0.732**	1.053	**1.633**
2237	FEN1	1.368	**0.483**	1.076	**0.633**
3014	H2AFX	1.458	**0.344**	1.199	**0.378**
1647	GADD45A	**0.670**	1.460	0.824	**1.768**
1407	CRY1	**0.282**	**0.544**	1.317	1.406

Footnote as in Table 1.

expressions of FEN1 and H2AFX were up-regulated at 8 h after irradiation in Seg-1 and Seg-1R cells comparing with the expression at 0 h, but up-regulated at 24 h after irradiation. GADD45A increased at 24 h after irradiation while decreased at 8 h after irradiation in both Seg-1 and Seg-1R cells.

Three genes, SLK, HMGB1 and PMS1, especially PMS1, not only differentially expressed in radiosensitive cells and radioresistant cells, but also showed significant differences at different time points (Figure 3).

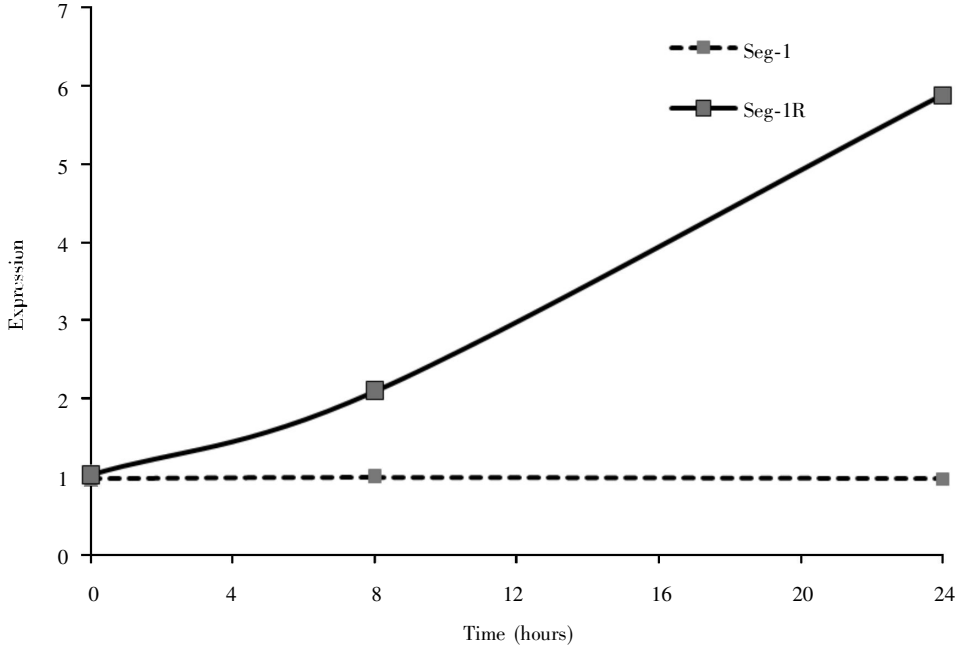

Figure 3 The expression tendency of PMS1 gene between Seg-1 and Seg-1R cells

The expression data of PMS1 are original values of genechips. The expression profiles of PMS1 did not show significantly changes between 8 h and 24 h after irradiation comparing with 0 h in Seg-1 cell lines, but PMS1 expresion was upregulated at 8 h and 24 h after irradiation in Seg-1R cells.

RT-PCR analysis

The results of RT-PCR analysis showed that the expression of PMS1 was significantly different between radiosensitive and radioresistant cells (Figure 4), which confirmed to microarray data.

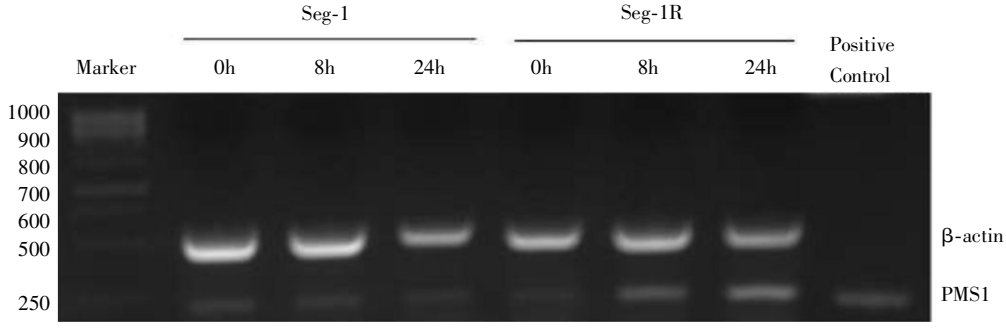

Figure 4 Expression of PMS1 in Seg-1 and Seg-1R cells detected by RT-PCR at different time points after irradiation

The result of RT-PCR was consistent with that of microarray. The expression of PMS1 changed slightly in Seg-1 cells at different time points after irradiation, but increased persistently in Seg-1R cells at 8 h and 24 h after irradiation.

Repetitive verification

We compared the expression of these ten genes between before irradiation and at 8 h and 24 h after irradiation, and then found that Kyse170 and Kyse170R cells were consistent with Seg-1 and Seg-1R cells in the changes of PMS1 expression at different time points after

irradiation (Figure 5). However, the expression of other genes did not show the same consistency.

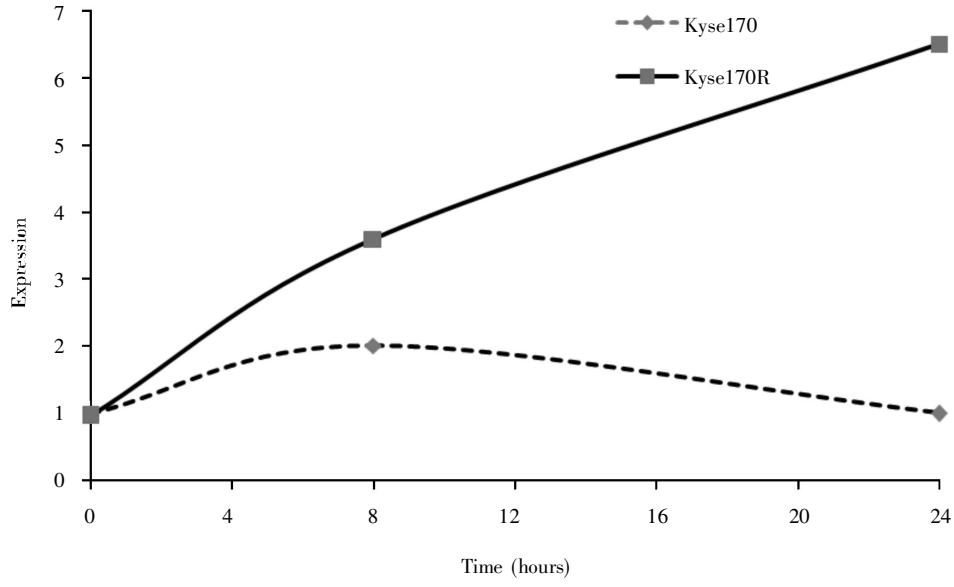

Figure 5 The expression tendency of PMS1 gene in Kyse170 and Kyse170R cells

The expression data of PMS1 are original values of genechips. The expression profiles of PMS1 did not show significant changes between 8 h and 24 h after irradiation comparing with 0 h in Kyse170 cell lines. However, PMS1 expression increased remarkably in Kyse170R cell lines after irradiation. The results from Kyse170 and Kyse170R cells were consistent with those from Seg-1 and Seg-1R cells.

Discussion

Radioresistance of cancer cells has become a hot issue in radiotherapy. Many factors affect radioresistance of cancer cells with unknown molecular mechanism. Abundant evidence showed that nuclear was the sensitive locus in the process of cell death causing by irradiation. Both single- and double-stranded DNA break after X-ray irradiation. Double-stranded DNA broken is considered to be the most important damage on chromosome, causing cell death. Homologous and non-homologous recombination can repair DNA damage[5]. Recently, almost all studies on DNA damage repair associated genes which play roles in radioresistance focus on analyzing the function of single gene[6-8], or screening differentially expressed genes without irradiation by microarray[9,10]. In the present study, we used whole human genome microarray with the characteristics of high-throughput, micromation and automation to screen DNA damage repair associated genes in esophageal cancer cells with or without irradiation treatment.

We further analyzed the variation of gene expression before and after irradiation. According to Gene Ontology analysis and molecular function, we found that ten DNA damage repair associated genes were differentially expressed between radiosensitive and radioresistant cancer cells as among well as three time points after irradiation in the same cell line. The expressions of SLK, HMGB1 and PMS1 showed significant differences between radiosensitive and radioresistant cancer cells. In addition, the expression of these genes, especially that of PMS1, was significantly different among three time points in the same cell line.

SLK plays an important role in cell repair and motility. Forte *et al.*[13] established a model of arteriotomy-induced stenosis in rat carotid arteries and found that the expression of DNA damage repair genes, Muyth and SLK, increased from 4 h to 7 days after arteriotomy by using RT-PCR and Western blot analysis. Additionally, Roovers *et al.*[14] showed that SLK was involved in the control of cell motility through its effects on actin protein reorganization and focal adhesion turnover and further indicated that SLK gene activated by over-expressed Neu gene can regulate the cell migration of mammary epithelia cancer cells in the presence of the Neu activator. In the present study, we showed that the expression of SLK significantly decreased from 0 h to 8 h after irradiation in radiosensitive cells, but increased at 24 h after irradiation. However, the expression of SLK increased significantly at 24 h after irradiation in radioresistant cells, while did not show significant increase at 0 h and 8 h after irradiation, indicating that SLK may be involved in DNA damage repair after irradiation.

HMGB1 has been found to up-regulate in many types of cancers, promoting tumor progression. On the other hand, HMGB1 activates dendritic cells and triggers anti-neoplastic response of T-cells in chemotherapy and radiotherapy [15]. Lange *et al.* [16] demonstrated that mammalian cells lacking HMGB1 were hyper-sensitive to DNA damage induced by psoralen plus UVA irradiation (PUVA) or UVC irradiation, showing less survival and increased mutagenesis. Furthermore, due to the absence of HMGB1, nucleotide excision repair efficiency was significantly decreased [16]. Yuan *et al.* [17] presented evidence that HMGB1 interacted physically with MutSa and was required at or prior to excision of the misrepaired nucleotide during DNA mismatch repair. In this study, the expression of HMGB1 decreased quickly from 0 h to 8 h after irradiation in radiosensitive cells, but no changes were found from 8 h to 24 h after irradiation. However, the expression of HMGB1, similar to that of SLK, significantly increased in radioresistant cells from 8 h to 24 h after irradiation. Therefore, both our results and previous studies indicated that HMGB1 may play essential roles in DNA damage repair after irradiation.

PMS1 encodes a protein of DNA repair family mutL/hexB and takes part in DNA repair by forming heterodimers with other DNA repair proteins [18]. Hall *et al.* [19] showed that heterodimers exhibited a much higher affinity for long DNA molecules and the rate of associatition with DNA was much more rapid than dissociation of protein-DNA complexes. Moreover, PMS1 binds with two different regions of double-stranded DNA simultaneously [19]. Chen *et al.* [20] found that PMS1 was only expressed in the basal cells in normal glands, whereas it was absent in prostate carcinoma cell lines DU145, LNCaP, p69SV40T, M2182 and M12. In the present study, we showed the expression of PMS1 changed significantly among all DNA damage repair associated genes. More importantly, the expression variation of PMS1 was different between radiosensitive cells and radioresistant cancer cells. The expression of PMS1 did not show significant changes between Seg-1 cells and Seg-1R cells without irradiation. After X-ray irradiation, PMS1 expression increased in radioresistant cancer cells and reached to 6.053 times 24 h after irradiation comparing with PMS1 expression at 0 h. The results from Kyse170 and Kyse170R cells were consistent with those from Seg-1 and Seg-1R cells. Taken together, our results implied that PMS1 was involved in double-stranded DNA damage repair through continuous increase of expression after X-ray irradiation, suggesting that PMS1 may be an important factor for radioresistance of esophageal cancer cells.

In summary, we screened ten DNA damage repair associated genes, which were related to radioresistance of esophageal cancer cells, especially PMS1. Further studies on these genes will be helpful for exploring the molecular mechanism of redioresistance of esophageal cells and providing powerful evidence for clinical prediction of radioresistance.

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