Original Article

Biofunctional study on chemoresistance in esophageal squamous carcinoma cells induced by missense mutation of *NOTCH1 p.E450K*

Keting Li[1#], Wentao Hao[2#], Jiwei Wu[1], Xianben Liu[1], Wenqun Xing[1], Yan Zheng[1]

[1]Department of Thoracic Surgery, The Affiliated Cancer Hospital of Zhengzhou University/Henan Cancer Hospital, Zhengzhou, China; [2]Department of Thoracic Surgery, Shanghai Public Health Clinical Center, Shanghai, China

Contributions: (I) Conception and design: J Wu, W Hao, K Li, Y Zheng; (II) Administrative support: X Liu, W Xing; (III) Provision of study materials or patients: W Xing, X Liu; (IV) Collection and assembly of data: W Hao, K Li; (V) Data analysis and interpretation: W Hao, K Li, Y Zheng; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work as co-first authors.

Correspondence to: Yan Zheng, MD, PhD. Department of Thoracic Surgery, The Affiliated Cancer Hospital of Zhengzhou University/Henan Cancer Hospital, 127 Dongming Road, Zhengzhou 450008, China. Email: sunnyzheng1@126.com.

Background: Neoadjuvant chemotherapy (nCT) combined with surgery is one of the main strategies for the treatment of resectable locally advanced esophageal squamous cell carcinoma (ESCC). However, nearly 40% of patients did not benefit from nCT, and the detection rate of *NOTCH1* missense mutation was significantly increased in patients who did not respond to chemotherapy, suggesting that the missense mutation may be related to tumor chemoresistance. We aim to explore the effect of a *NOTCH1* missense mutation on cell phenotype, to interpret the biofunctional changes in cell lines with a *NOTCH1* missense mutation and to analyze the effect of a *NOTCH1* missense mutation on drug resistance in ESCC cell lines.

Methods: Sanger sequencing was used to evaluate the exon mutations in the *NOTCH1* ligand binding region of candidate ESCC cell lines. After screening, KYSE450 and KYSE140 cells were selected as the research objects, and point mutation cell lines [KYSE140-mutant-type (MT) and KYSE450-MT] were constructed by CRISPR/Cas9 technology. Then, functional experiments were performed with the four cell lines [KYSE450-MT/wild-type (WT) and KYSE140-MT/WT]. The drug resistance of ESCC cell lines was assessed with a drug sensitivity test, and the proliferation, invasion and migration of ESCC lines were evaluated by proliferation test, scratch test and Transwell test. The cell cycle status of ESCC cells was assessed using flow cytometry.

Results: Drug sensitivity tests showed that the *NOTCH1 p.E450K* point mutation caused chemotherapy resistance in KYSE140 and KYSE450 ESCC cell lines. Cell proliferation, Wound scratch and Transwell assays showed that the *NOTCH1 p.E450K* point mutation enhanced the proliferation, invasion and migration abilities of KYSE140 and KYSE450 cells. Flow cytometry analysis showed that the *NOTCH1 p.E450K* point mutation caused an increase in KYSE140 and KYSE450 cells in S phase.

Conclusions: The *NOTCH1 p.E450K* point mutation causes chemotherapy resistance in KYSE140 and KYSE450 ESCC cells. Cell functional experiments showed that the *NOTCH1 p.E450K* point mutation enhanced the proliferation, migration and invasion abilities of KYSE140 and KYSE450 cells and increased the number of cells in S phase.

Keywords: *NOTCH1*; esophageal squamous cell carcinoma (ESCC); chemotherapy resistance

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Introduction

Esophageal cancer is a common malignant tumor of the gastrointestinal tract. In 2020, the incidence and mortality of esophageal cancer worldwide ranked eighth and sixth, respectively, among all malignant tumors (1). The main pathological types of esophageal cancer are esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC). More than half of ESCC cases worldwide are in China (2).

Neoadjuvant chemotherapy (nCT) combined with surgery is one of the main strategies for the treatment of resectable locally advanced ESCC. However, nearly 40% of patients do not benefit from nCT, and approximately 5% even experience progression (3), suggesting that these patients may have drug resistance mutations. In response to the chemotherapy resistance of some tumors, many scholars have conducted screening studies on drug-resistant mutations in the hope of improving the efficacy of chemotherapy (4). Some studies have shown that the abnormal activation of the *NOTCH* signaling pathway is closely associated with the occurrence, development, invasion, and metastasis of many tumors (5,6). It has been confirmed in esophageal cancer and other tumors that the activation of the *NOTCH1/HES1* pathway can lead to tumor chemotherapy resistance (7,8). However, most of these studies addressed the regulation of *NOTCH1* transcription or expression and the subsequent abnormal activation of the *NOTCH1/HES1* pathway leading to drug resistance. To date, no studies have shown that *NOTCH1* missense mutations affect the ligand binding status of receptors in this pathway to cause drug resistance. A previous study (9) on resistance to nCT in esophageal cancer showed that the detection rate of *NOTCH1* missense mutations was significantly increased in patients with ineffective nCT. Further study showed that *NOTCH1 p.E450K* was the only mutation that was being repeatedly detected. Our previous study showed that the *NOTCH1 p.E450K* (p.Glu450Lys) missense mutation may lead to tighter binding of the mutant protein to the DLL4 receptor, thereby continuously activating downstream signaling pathways (10). Missense mutations may alter drug effects by affecting cell signaling pathways. Based on this, we used a series of experiments to investigate the effect of a *NOTCH1* missense mutation on cell phenotype, to elucidate the biological and functional changes in cell lines with a *NOTCH1* missense mutation and to systematically analyze the effect of the *NOTCH1 p.E450K* missense mutation on the drug resistance of ESCC cells. We present this article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-23-1880/rc).

Highlight box

Key findings

- The *NOTCH1 p.E450K* point mutation causes chemotherapy resistance in KYSE140 and KYSE450 esophageal squamous cell carcinoma (ESCC) cells.

What is known and what is new?

- The detection rate of *NOTCH1* missense mutations was significantly increased in patients with ineffective neoadjuvant chemotherapy (nCT). Further study showed that *NOTCH1 p.E450K* was the only mutation that was being repeatedly detected.
- We elucidate the biological and functional changes in cell lines with a NOTCH1 missense mutation and systematically analyze the effect of the *NOTCH1 p.E450K* missense mutation on the drug resistance of ESCC cells.

What is the implication, and what should change now?

- ESCC is a malignant tumor with high incidence and strong invasiveness. nCT combined with surgery is one of the main strategies for the treatment of resectable locally advanced ESCC. Although nCT has achieved good effects, resistance of tumor cells to chemotherapy is still a problem. If the mechanism underlying the involvement of the *NOTCH* signaling pathway in cell drug resistance is elucidated, it may provide new therapeutic targets and targeted pathways for molecular targeted therapy for the reversal of drug resistance in esophageal cancer. This missense mutation may be a potential predictor of clinical chemotherapy efficacy and an important target for the reversal of drug resistance, which is worthy of further study.

Methods

Cell lines and Sanger sequencing

ESCC cell lines KYSE140, KYSE150, KYSE410, KYSE450, and KYSE510 (from the Hormel Research Institute of Henan Cancer Hospital, a donation from Professor Kangdong Liu) were chosen as candidate cells. The DNA of relevant cells was extracted and subjected to polymerase chain reaction (PCR) amplification and electrophoresis, and after purification, sequencing screening was performed. According to the sequencing results, KYSE140 and KYSE450 cells were selected as the research objects of this experiment. Sanger sequencing was performed at BGI Genomics (Shenzhen, China).

Construct of point mutation cell lines

The KYSE140 cell line and KYSE450 cell line were

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chosen as the research objects of this study, and CRISPR/Cas9 technology was used to establish the KYSE140 and KYSE450 point mutation cell lines. This experiment was partly completed by Beijing Biocytogen Biotechnology Co., Ltd. (Beijing, China).

Construction of a point mutation cell line was first conducted using the KYSE140 cell line (*Figure 1*). The point mutation c.1348G>A (pGlu450Lys) was knocked-in to exon 8 of the *NOTCH1* gene. After target sequencing was confirmed, the single-guide RNA (sgRNA) recognition sequence was completely consistent with the DNA sequence of the KYSE140 cell line. According to the design principles of sgRNA, at the 5' target locus, eight sgRNAs were designed for the 3' target site region. After validation, sgRNA5 and sgRNA12 were selected and electrotransformed into the KYSE140 cell line with the targeting vector. After drug screening, positive clone enrichment, and PCR screening, two positive clones were obtained. Then, PCR product sequencing confirmed that the KYSE140 cell line was homozygous for the knock-in point mutation. The KYSE450 point mutation cell line was prepared using the same approach as that used for the KYSE140 cell line, and two positive clones were obtained.

Drug sensitive test

The above groups of cells in the logarithmic growth phase were digested by trypsin (Servicebio G4004, Wuhan, China), seeded in 96-well plates at 5×10^3 cells per well, and incubated for 12 h. Then, cell suspensions were treated with cisplatin (0, 3.1, 6.3, 13, 25, 50 μg/mL) or paclitaxel (0.8, 1.6, 3.1, 6.2, 12.5, 25, 50, 100, 200 μg/mL). After 0, 24, and 48 h of treatment, 10 μL of Cell Counting Kit-8 (CCK-8) reagent (Vazyme, Nanjing, China) was added to each well. After the addition of the CCK-8 reagent, the cells were incubated for another 4 h. Optical density (OD) was measured with a microplate reader (Perlong, Beijing, China). Cell survival rate was calculated as (OD value of experimental group – OD value of blank group)/(OD value of control group – OD value of blank group) × 100%. The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 5.0 software to study and evaluate the sensitivity and resistance of cells to cisplatin/paclitaxel.

Wound scratch assay

A marker pen and ruler were irradiated with UV light for 30 min on a clean bench. A horizontal line was drawn on the back of each six-well plate, and approximately 1×10^5 cells were added to reach confluence overnight. The cell layer was scratched on the second day. After scratching, the cell layer was washed with phosphate-buffered saline (PBS) and incubated in fresh serum-free culture medium in an incubator (37 ℃, 5% CO_2). Then, the cells were removed from the incubator at 0 and 24 h and observed under a microscope (quadruple field of view). The scratches were photographed, and the area of the scratch was calculated (UOP DSY5000X, USA). After opening the images in ImageJ, eight horizontal lines were randomly drawn, and the mean value of intercellular distances was calculated.

Transwell cell migration and invasion assay

Cells were digested with trypsin, counted, and adjusted to an appropriate density. In a 24-well Transwell plate (NEST 0223A, China), 400 μL of complete medium was added to the lower chamber, and 200 μL of serum-free cell suspension (NEST 725301, China) was added to the upper chamber. After culturing for 24 h in an incubator, the cells were fixed in paraformaldehyde for 25 min, washed three times with 0.9% NaCl, and stained with crystal violet for 40 min. A multifunctional camera was used to take pictures, and differences between the groups were observed. The measurement values were recorded, and the results were analyzed using GraphPad Prism 5.0 software.

Cell proliferation assay

Cell proliferation was evaluated by the CCK-8 assay. A total of 1×10^3 KYSE140-wild-type (WT)/mutant-type (MT) or KYSE450-WT/MT cells were seeded in each well of a 96-well plate. After overnight incubation, absorbance value was read at a reference of 450 nm. The inhibition rate (%) was calculated as (1 – OD value of treated group/OD value of the control group) × 100%.

Flow cytometry

Propidium iodide staining was used to assess the cell cycle status of the ESCC cell lines in the absence and presence of the *NOTCH1 p.E450K* point mutation. Cells in each group were digested with trypsin, centrifuged and fixed in 75% ethanol, then, propidium iodide staining solution was added and the cells were incubated at room temperature for 30 min in the dark, stored at 4 ℃ in the dark and analyzed within 24 h. A flow cytometer (BD Biosciences Canto II,

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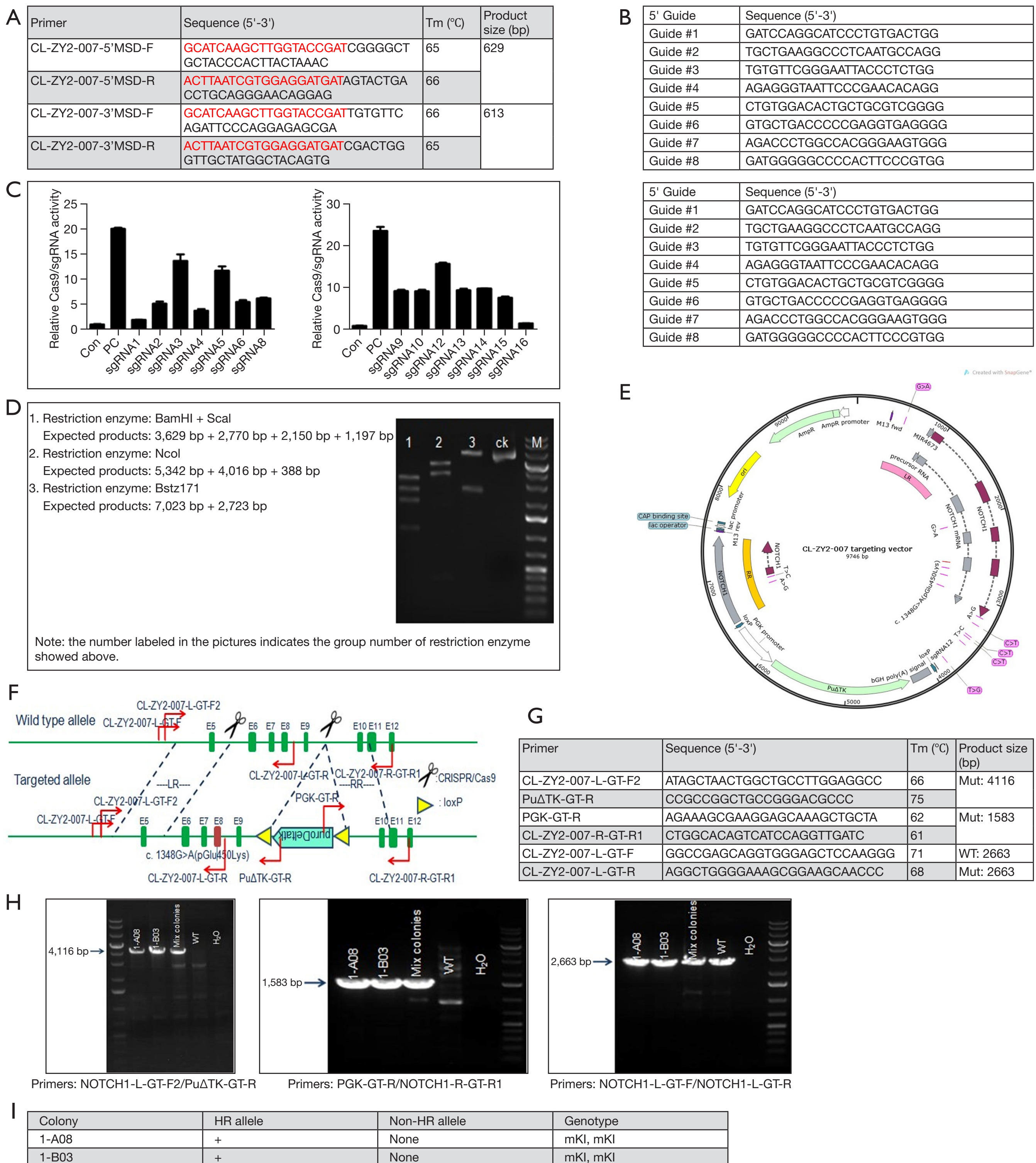

A

Primer	Sequence (5'-3')	Tm (℃)	Product size (bp)
CL-ZY2-007-5'MSD-F	GCATCAAGCTTGGTACCGATCGGGGCT GCTACCCACTTACTAAAC	65	629
CL-ZY2-007-5'MSD-R	ACTTAATCGTGGAGGATGATAGTACTGA CCTGCAGGGAACAGGAG	66	
CL-ZY2-007-3'MSD-F	GCATCAAGCTTGGTACCGATTGTGTTC AGATTCCCAGGAGAGCGA	66	613
CL-ZY2-007-3'MSD-R	ACTTAATCGTGGAGGATGATCGACTGG GTTGCTATGGCTACAGTG	65	

B

5' Guide	Sequence (5'-3')
Guide #1	GATCCAGGCATCCCTGTGACTGG
Guide #2	TGCTGAAGGCCCTCAATGCCAGG
Guide #3	TGTGTTCGGGAATTACCCTCTGG
Guide #4	AGAGGGTAATTCCCGAACACAGG
Guide #5	CTGTGGACACTGCTGCGTCGGGG
Guide #6	GTGCTGACCCCCGAGGTGAGGGG
Guide #7	AGACCCTGGCCACGGGAAGTGGG
Guide #8	GATGGGGGCCCCACTTCCCGTGG

5' Guide	Sequence (5'-3')
Guide #1	GATCCAGGCATCCCTGTGACTGG
Guide #2	TGCTGAAGGCCCTCAATGCCAGG
Guide #3	TGTGTTCGGGAATTACCCTCTGG
Guide #4	AGAGGGTAATTCCCGAACACAGG
Guide #5	CTGTGGACACTGCTGCGTCGGGG
Guide #6	GTGCTGACCCCCGAGGTGAGGGG
Guide #7	AGACCCTGGCCACGGGAAGTGGG
Guide #8	GATGGGGGCCCCACTTCCCGTGG

G

Primer	Sequence (5'-3')	Tm (℃)	Product size (bp)
CL-ZY2-007-L-GT-F2	ATAGCTAACTGGCTGCCTTGGAGGCC	66	Mut: 4116
PuΔTK-GT-R	CCGCCGGCTGCCGGGACGCCC	75	
PGK-GT-R	AGAAAGCGAAGGAGCAAAGCTGCTA	62	Mut: 1583
CL-ZY2-007-R-GT-R1	CTGGCACAGTCATCCAGGTTGATC	61	
CL-ZY2-007-L-GT-F	GGCCGAGCAGGTGGGAGCTCCAAGGG	71	WT: 2663
CL-ZY2-007-L-GT-R	AGGCTGGGGAAAGCGGAAGCAACCC	68	Mut: 2663

I

Colony	HR allele	Non-HR allele	Genotype
1-A08	+	None	mKI, mKI
1-B03	+	None	mKI, mKI

Figure 1 Construction of KYSE140 point MT cell lines. (A) Target sequence sequencing confirmation; (B) Cas9/sgRNA design; (C) Cas9/sgRNA activity detection; (D) after enzyme digestion identification and sequencing, it was confirmed that the construction of the target vector was completed; (E) cell targeting vector map; (F) illustration of the design principles of identification primers; (G) identification of primer information; (H) part of the identification results; (I) positive clones are summarized. Tm, temperature; F, forward; R, reverse; Con, control; PC, positive control; sgRNA, single-guide RNA; HR, homologous recombination; mKI, mut knockin; MT, mutant-type; WT, wild-type.

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San Jose, CA, USA) was used to detect red fluorescence at an excitation wavelength of 535 nm and an emission wavelength of 615 nm and light scatter. Cellular DNA content analysis and light scatter analysis were performed using FlowJo analysis software (FlowJo10.2).

The apoptosis rates of the ESCC cell lines in the absence and presence of the *NOTCH1 p.E450K* point mutation were determined using an Annexin V-fluorescein isothiocyanate (V-FITC)/propidium iodide (PI) kit (Beyotime C1062S, Shanghai, China). Briefly, the cells were digested, washed, and centrifuged in cold PBS. Staining was performed with Annexin V-FITC and PI at room temperature (15 min). Flow cytometry (BD Biosciences Cantoll) was performed to analyze cell apoptosis. Data were quantified using FlowJo analysis software (FlowJo10.2).

Statistical analysis

The data were statistically analyzed and compared using the statistical software SPSS 23.0 (IBM, Armonk, NY, USA). Each experiment was repeated at least three times, and $P<0.05$ was considered statistically significant.

Ethical statement

The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was reviewed and approved by the Ethics Review Committee of the Affiliated Cancer Hospital of Zhengzhou University/Henan Cancer Hospital (No. 2021-KY-0049-001).

Results

Sanger sequencing screening

Sequencing results showed that the *NOTCH1 p.E450K* point mutation was located in *NOTCH1*-E8 and that KYSE140 and KYSE450 cells did not have exon mutations in the *NOTCH1* ligand binding region in E8. KYSE140 and KYSE450 cells were chosen as the research objects of this study.

Construction of point MT cell lines (CRISPR/Cas9 technology)

The *NOTCH1* gene is approximately 51.4 kb in length and is located on the anti-strand on chromosome 9 (NCBI ID: 4851). After drug screening, the enrichment of positive clones, and PCR screening, two positive clones were finally obtained. PCR product sequencing showed that 1-A08, 1-B03, 1-A01, and 1-B12 were homozygous knock-in clones of the KYSE140/450 cell lines. The construction and validation of the *NOTCH1* c.1348G>A (pGlu450Lys) point mutation in the two cell lines are shown in *Figure 2*.

NOTCH1 p.E450K point mutation promoted chemotherapy resistance

To study the effect of the *NOTCH1 p.E450K* point mutation on chemotherapy resistance in ESCC cell lines, the KYSE140/450 cell lines were treated with cisplatin or paclitaxel.

The inhibition results for the KYSE140-WT/MT and KYSE450-WT/MT cell lines after treatment with different concentrations of cisplatin showed that with increasing concentrations of cisplatin, the inhibitory effect on the KYSE140-WT/MT cell line and the KYSE450-WT/MT cell line gradually increased in a dose-dependent manner, the IC_{50} value of the MT cell line KYSE140-MT/KYSE450-MT was higher than that of the WT cell line KYSE140-WT/KYSE450-WT. Under the same cisplatin concentration, the inhibitory effect of cisplatin on the MT cell line was always weaker than that on the WT cell line (*Figure 3A,3B*), and the difference was significant ($P<0.001$). This suggests that the *NOTCH1 p.E450K* point mutation can reduce the sensitivity of ESCC cell lines to chemotherapeutic agents, which in turn leads to chemoresistance, in terms of IC_{50}, the same conclusion was obtained in the paclitaxel-treated group (*Figure 3C,3D*).

NOTCH1 p.E450K point mutation enhanced the invasion and migration abilities of ESCC cell lines

Through scratch experiments and Transwell experiments, the changes in the migration, invasion and metastasis of the KYSE140/450 cell lines under the effect of the *NOTCH1 p.E450K* point mutation were investigated. The results of the scratch experiment showed that the *NOTCH1 p.E450K* point mutation resulted in the enhanced invasion and migration abilities of KYSE140 and KYSE450 cells (*Figure 4A,4B*). Transwell experiments showed that the *NOTCH1 p.E450K* point mutation enhanced the invasiveness of the KYSE140 and KYSE450 cell lines (*Figure 4C,4D*).

NOTCH1 p.E450K point mutation promoted proliferation

To figure out the effect of *NOTCH1 p.E450K* point

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I

Colony	HR allele	Non-HR allele	Genotype
1-A08	+	None	mKI, mKI
1-B03	+	None	mKI, mKI

J

Colony	HR allele	Non-HR allele	Genotype
1-A01	+	None	mKI, mKI
1-B12	+	None	mKI, mKI

Figure 2 CRISPR/Cas9 technology to construct *NOTCH1 p.E450K* point MT KYSE140 and KYSE450 esophageal cancer cell lines. (A,C,E,G,I) Illustration of the construction and validation of missense MT cells of the KYSE140 cell line; (B,D,F,H,J) illustration of the construction and validation of missense MT cells of the KYSE450 cell line. (A,B) Schematic diagrams of targeting based on CRISPR/Cas9 technology; (A) knock-in point mutation c.1348G>A (pGlu450Lys) in exon 8 of CL-ZY2-007 gene; (B) knock-in point mutation c.1348G>A (pGlu450Lys) in exon 8 in CL-ZY2-008 gene; (C,D) the cell target vector map; (E,F) the design of positive clone screening primers; (G,H) the PCR positive clone screening results; (I,J) the summary of PCR product sequencing results; (G,I) 1-A08 and 1-B03 are the KYSE140 cell line *NOTCH1* point mutation homozygous knock-in positive clones; (H,J) 1-A01 and 1-B12 are KYSE450 cell line point mutation homozygous knock-in positive clones. *Figure 2C* excerpted *Figure 1E*; *Figure 2E* excerpted *Figure 1F*; *Figure 2G* excerpted the terminal two panels of *Figure 1H*; and *Figure 2I* excerpted *Figure 1I*. HR, homologous recombination; mKI, mut knockin; MT, mutant-type; PCR, polymerase chain reaction.

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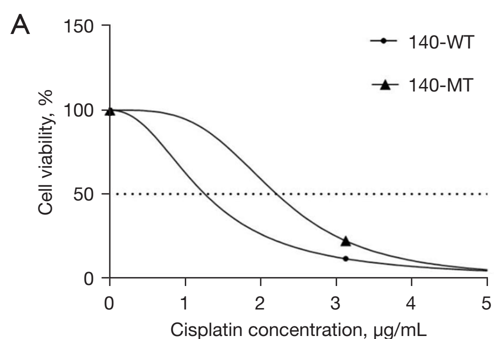
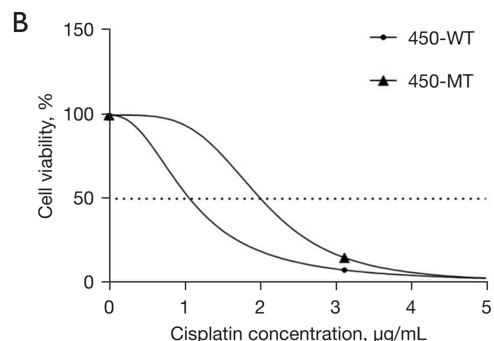
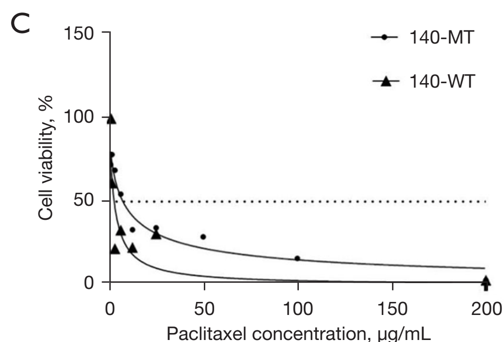


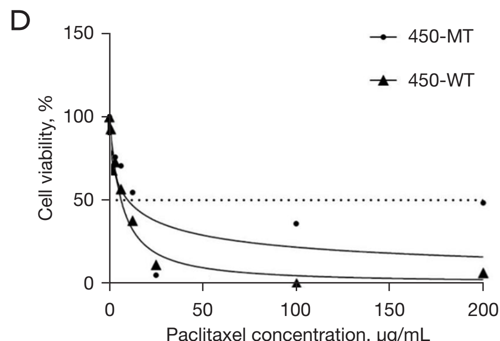
[Inhibitor] *vs.* normalized response—variable slope [2]	
Best-fit values	
IC_{50}	1.260
Hillslope	−2.245
$LogIC_{50}$	0.1005
95% CI (profile likelihood)	
IC_{50}	0.04612 to + infinity
Hillslope	−infinity to −0.5994
$LogIC_{50}$	−1.336 to ???
Goodness of fit	
Degrees of freedom	16
R squared	0.9855
Sum of squares	347.7
Sy.x	4.661
Constraints	
IC_{50}	IC_{50} >0
Number of points	
# of X values	18
# Y values analyzed	18



[Inhibitor] *vs.* normalized response—variable slope [2]	
Best-fit values	
IC_{50}	1.074
Hillslope	−2.343
$LogIC_{50}$	0.03118
95% CI (profile likelihood)	
IC_{50}	0.07376 to + infinity
Hillslope	−infinity to −0.7649
$LogIC_{50}$	−1.132 to ???
Goodness of fit	
Degrees of freedom	16
R squared	0.9950
Sum of squares	121.4
Sy.x	2.755
Constraints	
IC_{50}	IC_{50} >0
Number of points	
# of X values	18
# Y values analyzed	18



[Inhibitor] *vs.* normalized response—variable slope [2]	
Best-fit values	
IC_{50}	7.451
Hillslope	−0.6584
$LogIC_{50}$	0.8722
95% CI (profile likelihood)	
IC_{50}	4.840 to 11.20
Hillslope	−0.8814 to −0.4844
$LogIC_{50}$	−0.6848 to 1.049
Goodness of fit	
Degrees of freedom	8
R squared	0.9581
Sum of squares	366.3
Sy.x	6.767
Constraints	
IC_{50}	IC_{50} >0
Number of points	
# of X values	10
# Y values analyzed	10



[Inhibitor] *vs.* normalized response—variable slope [2]	
Best-fit values	
IC_{50}	9.936
Hillslope	−0.5548
$LogIC_{50}$	0.9972
95% CI (profile likelihood)	
IC_{50}	0.8264 to 96.13
Hillslope	−3.220 to −0.08497
$LogIC_{50}$	−0.08283 to 1.983
Goodness of fit	
Degrees of freedom	8
R squared	0.6243
Sum of squares	3539
Sy.x	21.03
Constraints	
IC_{50}	IC_{50} >0
Number of points	
# of X values	10
# Y values analyzed	10

Figure 3 Results of cell viability detection in each group after different concentrations of chemotherapy drugs. (A,B) Cell viability curve in each group after different concentrations of cisplatin. (C,D) Cell viability curve in each group after different concentrations of paclitaxel. WT, wild-type; MT, mutant-type; IC_{50}, half-maximal inhibitory concentration; CI, confidence interval.

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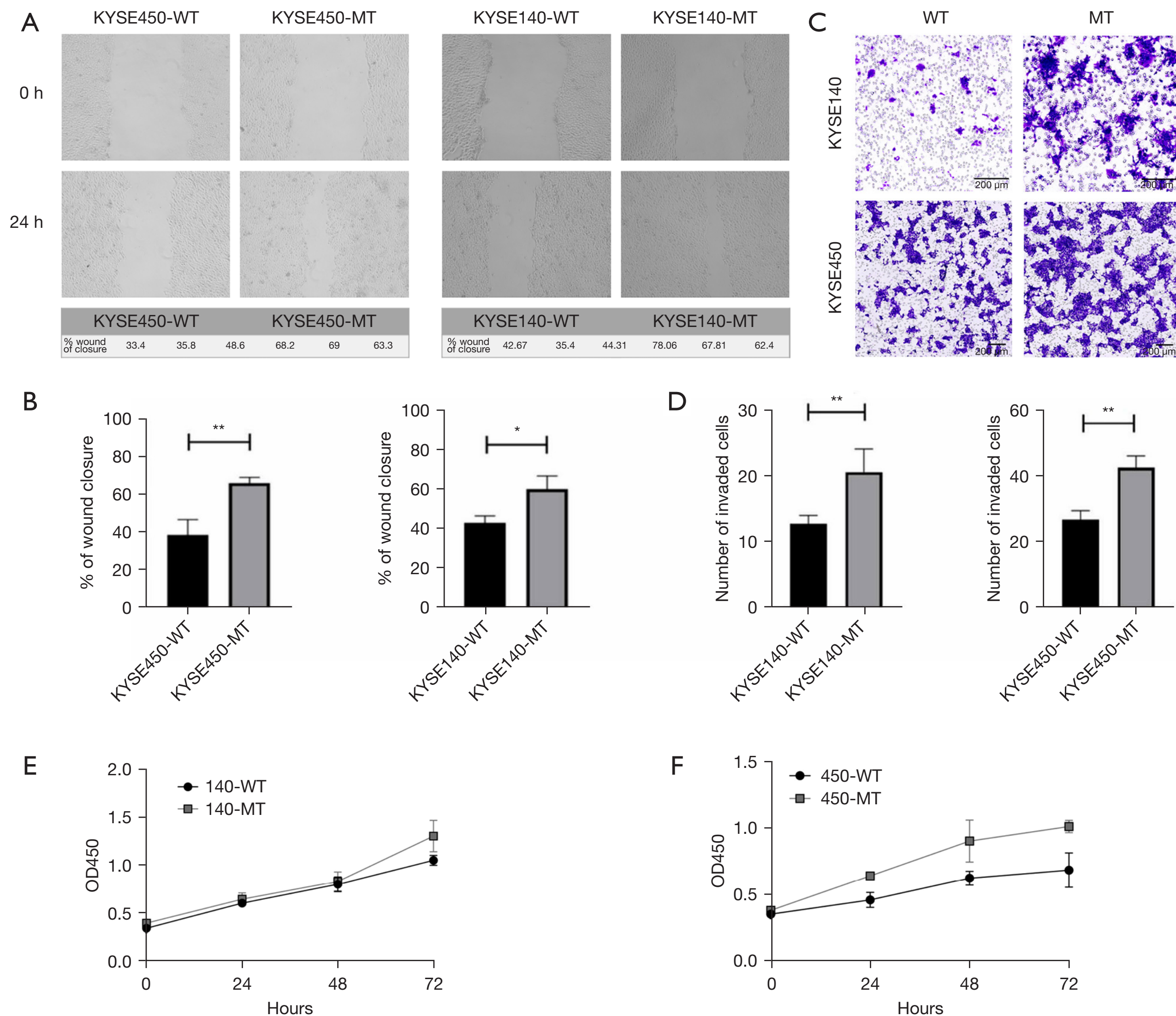

Figure 4 *NOTCH1 p.E450K* point mutation enhances the migration and invasion ability of KYSE140/KYSE450 esophageal cancer cells, and accelerates the proliferation. (A,B) Wound scratch assay showed that *NOTCH1 p.E450K* point mutation enhanced the invasion and migration ability of KYSE140/KYSE450 cells. (C,D) Transwell assay (crystal violet staining) showed that *NOTCH1 p.E450K* point mutation enhanced the invasion and migration ability of KYSE140/KYSE450 cells. (E,F) CCK-8 proliferation assay showed that no significant difference in the proliferation ability of KYSE140/KYSE450 cells before and after *NOTCH1 p.E450K* point mutation. *, P<0.05; **, P<0.01. WT, wild-type; MT, mutant-type; OD, optical density; CCK-8, Cell Counting Kit-8.

mutation on the proliferation, we compared WT esophageal cancer cell lines with MT esophageal cancer cell lines using CCK-8 assay. There was no statistical significance in the comparison of proliferative ability of ESCC cells before and after *NOTCH1 p.E450K* point mutation, indicating that point mutation had no significant effect on the proliferation of ESCC cells (*Figure 4E,4F*).

The effects of the point mutation on the cell cycle

Flow cytometry analysis showed that the *NOTCH1 p.E450K* point mutation caused a significant increase in the proportion of KYSE140 and KYSE450 cells in S phase (*Figure 5*). S phase is the main period of DNA synthesis, also known as the DNA replication phase, during which the DNA number doubles. In general, once cells enter S phase,

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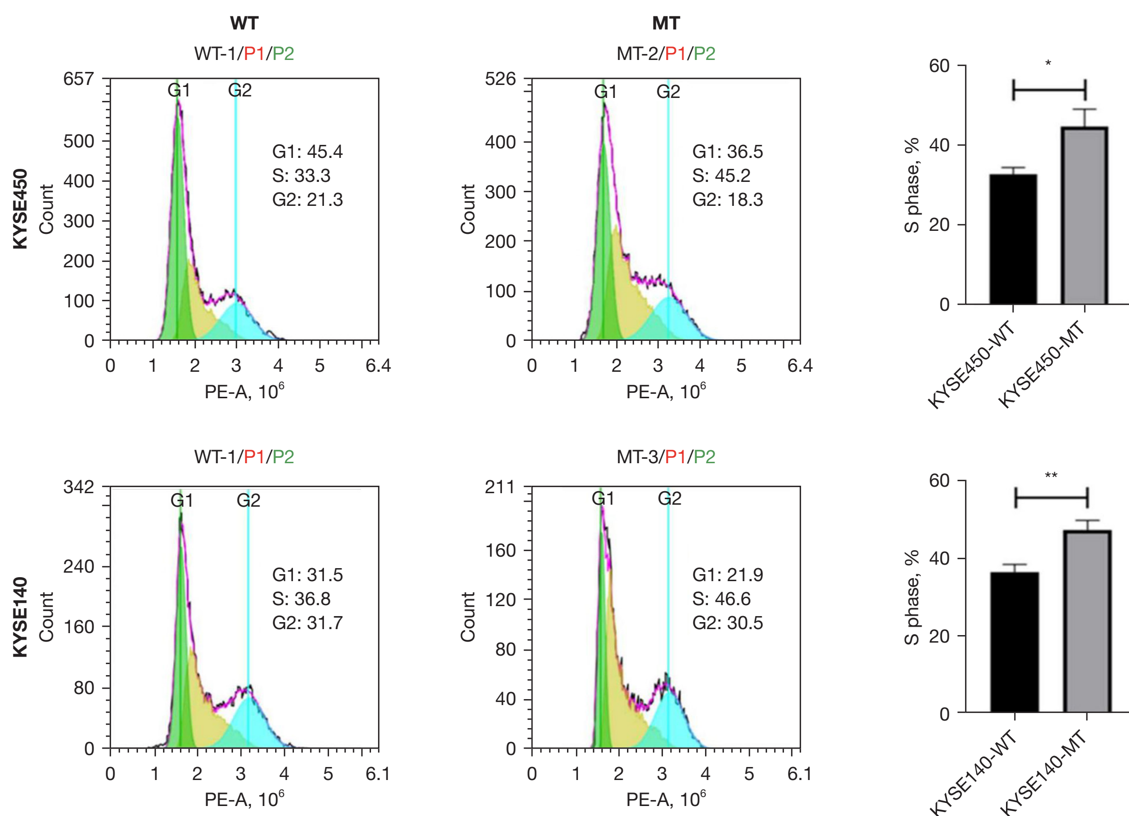

Figure 5 Effect of *NOTCH1 p.E450K* mutation on cell cycle of esophageal cancer cells. *NOTCH1 p.E450K* point mutation caused a significant increase in the proportion of S phase cells in KYSE140/KYSE450 cells. *, P<0.05; **, P<0.01. WT, wild-type; MT, mutant-type.

cell division continues until the G1 phase of the next cycle. An increase in cells in S phase indicates proliferation.

Discussion

Although it has been confirmed in many cancers, including esophageal cancer, that the activation of the *NOTCH1* pathway leads to drug resistance in malignant tumors (7), little is known about the *NOTCH* pathway causing drug resistance in ESCC. In previous reports on ESCC gene sequencing, the frequency of *NOTCH1* mutations was lower only than that of *p53* (11,12). Many studies (13-22) have explained how the activation of the *NOTCH1* signaling pathway leads to chemotherapy resistance. However, these studies mostly addressed the regulation of *NOTCH1* transcription or expression, followed by the abnormal activation of the *NOTCH1* pathway, which leads to chemotherapy resistance. No *NOTCH1* sense mutations have been found, nor reports of *NOTCH1* mutations that affect the ligand binding status of receptors in this pathway, resulting in chemotherapy resistance.

A previous study on drug resistance to nCT in esophageal cancer showed that in patients with ineffective chemotherapy, the detection rate of *NOTCH1* missense mutations was significantly increased (9). Further study found that the p.E450K missense mutation increased the binding ability of the *NOTCH1* receptor protein to its ligand DLL4 (10). On this basis, we investigated the effect of the *NOTCH1 p.E450K* missense mutation on cell phenotype to elucidate the biofunctional changes in NOTCH1 missense mutation cell lines. The results showed that the *NOTCH1 p.E450K* point mutation caused chemotherapy resistance in KYSE140 and KYSE450 ESCC cell lines, enhanced cell migration and invasion abilities, and increased the number of cells in S phase.

A study by Xie *et al.* (23) showed that high *NOTCH1* expression promoted resistance to epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) in non-small cell lung cancer (NSCLC). Zhang *et al.* (24) showed that the *NOTCH* signaling pathway was activated in the erlotinib-resistant NSCLC cell line HCC827/ER. In a

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breast cancer study, Pandya *et al.* (25) found that positive human epidermal growth factor receptor 2 (ErbB-2) expression inhibited the transcriptional activity of *NOTCH* and that herceptin treatment activated the *NOTCH* signaling pathway and led to chemotherapy resistance. In the T47D model of tamoxifen-resistant breast cancer, abnormally activated PKCa specifically upregulated the expression of *NOTCH* to cause drug resistance in breast cancer cells (26). Hang *et al.* (27) found that *NOTCH1* was highly expressed in cisplatin-resistant gastric cancer cell lines. Mirone *et al.* (28) found that *NOTCH1* was significantly highly expressed in colorectal cancer (CRC) cell lines that were resistant to regorafenib and that the downregulation of *NOTCH1* expression reversed drug resistance. Huang *et al.* (29) found that in CRC patients, *NOTCH1* was abnormally highly expressed in cells resistant to oxaliplatin and fluorouracil. Takam *et al.* (30) found that *NOTCH1* and the downstream target gene Hes1 of the *NOTCH* signaling pathway were highly expressed in human bone marrow mesenchymal stem cells (hBM-MSCs) from acute myeloid leukemia (AML) patients and that hBM-MSCs mediated and activated the NOTCH signaling pathway to promote the proliferation and drug resistance of leukemia cells. In chemotherapy sensitivity studies of breast cancer (31) and prostate cancer (32), it was found that the sensitivity of some patients to chemotherapeutic drugs could be improved by downregulating the *NOTCH1* signaling pathway, suggesting that the *NOTCH1* receptor and its ligand-mediated signaling pathways are closely related to chemotherapy resistance.

In the drug sensitivity test of the KYSE140 ESCC cell lines, the results showed that as the concentration of paclitaxel/cisplatin increased, its inhibitory effect on the KYSE140-WT and KYSE140-MT cell lines gradually increased (cell viability gradually decreased) in a dose-dependent manner. The IC_{50} results for paclitaxel/cisplatin showed that the IC_{50} concentration for the cells in the KYSE140-WT group was significantly lower than that for the cells in the KYSE140-MT group ($P<0.01$), indicating that signaling pathways in the KYSE140 cell line with the *NOTCH* missense mutation may be abnormally activated. Activation leads to the drug-resistant phenotype in tumor cells and the enhancement of chemotherapy resistance in ESCC cell lines. The same conclusion was obtained in the comparison of KYSE450-MT and KYSE450-WT cells.

The *NOTCH* signaling pathway is closely related to cell proliferation and differentiation and plays a very important regulatory role in body development. A number of studies have found that abnormal *NOTCH* signaling is associated with various common tumors, such as gastric cancer (33), breast cancer (34), lung cancer (35), and liver cancer (36). Some studies (37,38) also found that *NOTCH* signaling is involved in the process of tumor invasion and metastasis. A study on ESCC (7) explored the association between gene mutations in tumor cells and prognosis and chemotherapy response. The results showed that patients with *NOTCH1* mutations survived for a shorter time than patients without *NOTCH1* mutations and patients with *NOTCH1* mutations did not respond to chemotherapy. By comparing pathological staging among patients with HCC, Wang *et al.* (39) found that *NOTCH1* gene expression in patients with late-stage disease (stage III/IV) was higher than that in patients with earlier stage disease (stage I/II), suggesting that the *NOTCH1* gene expression level may be associated with the invasion and metastasis of hepatocellular carcinoma (HCC). Another study (40) found that Jagged1, a *NOTCH* signaling ligand, was associated with the recurrence and metastasis of prostate cancer. Some studies (41,42) have confirmed that the *NOTCH* pathway can synergize with other tumor signaling pathways, such as the *EGFR*, *Ras*, and *Akt* signaling pathways, which are very important for tumor development.

In this study, in the scratch and Transwell experiments, the MT cell line had stronger invasion and migration abilities than the WT cell line ($P<0.05$). Flow cytometry analysis also showed that the proportion of cells in S phase among the MT cells was significantly higher than that among WT cells, and proliferation test results showed that the proliferation ability of *NOTCH1* mutation cell line was enhanced. In general, once cells enter S phase, cell division continues until the G1 phase of the next cycle. An increase in the number of tumor cells in S phase indicates tumor cell proliferation and increases the ability of tumor cells to resist chemotherapy.

In the KYSE140 and KYSE450 ESCC cell lines, the *NOTCH1 p.E450K* point mutation enhanced the migration and invasion abilities and increased the percentage of cells in S phase. Although cell function experiments have yielded satisfactory results, they only reflect laboratory conditions, and therefore, further verification is needed by carrying out corresponding animal tumor model experiments. ESCC is a malignant tumor with high incidence and strong invasiveness. Although nCT has achieved good effects, resistance of tumor cells to chemotherapy is still a problem. If the mechanism underlying the involvement of the *NOTCH* signaling pathway in cell drug resistance is elucidated, it may provide new therapeutic targets and

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targeted pathways for molecular targeted therapy for the reversal of drug resistance in esophageal cancer. This missense mutation may be a potential predictor of clinical chemotherapy efficacy and an important target for the reversal of drug resistance, which is worthy of further study.

Conclusions

The *NOTCH1 p.E450K* point mutation causes chemotherapy resistance in KYSE140 and KYSE450 ESCC cells. Cell functional experiments showed that the *NOTCH1 p.E450K* point mutation enhanced the proliferation, migration and invasion abilities of KYSE140 and KYSE450 cells and increased the number of cells in S phase; the mutation had no effect on apoptosis.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was reviewed and approved by the Ethics Review Committee of the Affiliated Cancer Hospital of Zhengzhou University/Henan Cancer Hospital (No. 2021-KY-0049-001).

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