High miR-718 Suppresses Phosphatase and Tensin Homolog (PTEN) Expression and Correlates to Unfavorable Prognosis in Gastric Cancer

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Background: Phosphatase and tensin homolog (PTEN) is a kind of phosphatase which has been demonstrated to suppress progression of gastric cancer. Many micro-RNAs (miRNAs), such as miR-106b, miR-93, and miR-200c, could inhibit expression of PTEN in cell lines; and many miRNAs including miR-21, miR-22, miR-18a, and miR-222 are related to the progression and prognosis of gastric cancer. However, among these miRNAs, the clinical significance of miR-718 has not yet been elucidated.

Material/Methods: The expression of PTEN and miR-718 in 141 gastric cancer tissues were detected by immunohistochemistry and quantitative real-time PCR respectively. The correlation between PTEN, miR-718, and the clinicopathological factors was analyzed by χ² test. The prognostic significance of PTEN and miR-718 was evaluated by univariate and multivariate analysis. Luciferase reporter assay was performed to evaluate the regulation of PTEN by miR-718. The effect of miR-718 on gastric cancer proliferation and invasion was investigated by MTT assay and Transwell assay.

Results: Low expression of PTEN and high expression of miR-718 were both significantly associated with unfavorable prognosis, and both were identified as biomarkers predicting poorer prognosis of patients with gastric cancer. Increased miR-718 expression could decrease PTEN expression, thus enhancing phosphatidylinositide 3-kinases/protein kinase B (PI3K/Akt) signaling. Moreover, the abilities of proliferation and invasion of gastric cells transfected with miR-718 were promoted significantly compared with those transfected with control miRNA.

Conclusions: Low expression of PTEN and increased expression of miR-718 in gastric cancer tissues were both independent unfavorable prognostic factors of gastric cancer. Upregulation of miR-718 could increase PI3K/Akt signaling by directly downregulating PTEN, thus promoting the proliferation and invasion of gastric cancer cells.

MeSH Keywords: MicroRNAs • Prognosis • PTEN Phosphohydrolase • Stomach Neoplasms

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In our study, we detected the level of miR-718 as well as the expression of PTEN in gastric tumor tissues, and we analyzed their correlation with the survival rate. With experiments in vitro, we further detected whether miR-718 targeted PTEN directly in gastric cancer cells, and we explored the influence of miR-718 on the proliferation and invasion of gastric cancer.

Material and Methods

Patients and follow-ups

From 2006 to 2012, a total of 424 patients underwent radical resection of gastric adenocarcinoma in Yishui Central Hospital and Qianfoshan Hospital. The final diagnosis of gastric adenocarcinoma was confirmed by routine pathology. Then 141 patients were further enrolled following the criteria: 1) available tissue samples and medical records, 2) available follow-ups more than 5 months after operation, and 3) no severe perioperative complications and other tumors. Another 12 pairs of fresh tumor tissue and adjacent tissue were collected for the detection of PTEN mRNA level. All the samples were obtained with prior patients’ consents, and the approval of the Institutional Clinical Ethics Review Board of Yishui Central Hospital and Qianfoshan Hospital. The study was supervised by the Ethics Committee of Yishui Central Hospital and Qianfoshan Hospital. The tumor TNM stage was confirmed according to the guideline of 7th American Joint Committee on Cancer/Union for International Cancer Control.

Immunohistochemistry and evaluation

Sections of formalin-fixed, paraffin-embedded gastric cancer tissues were immunohistochemically (IHC) stained to evaluate the PTEN expression as previous described [18,19]. Briefly, the formalin-fixed and paraffin-embedded specimens were deparaffinized and rehydrated in graded ethanol. Optimal antigen retrieval was performed by heating in citrate buffer (pH = 6.0), and endogenous peroxidase activity was blocked by incubation in 5% fetal bovine serum, the primary antibody was then added and endogenous peroxidase activity was blocked by incubation in 5% fetal bovine serum, the primary antibody was then added and incubated at room temperature overnight. After washing with PBS, the slides were incubated with biotin-labeled secondary antibody and then with streptavidin-peroxidase. The aminoethyl-diamine reaction product was developed with diaminobenzidine. Sections were then dehydrated, cleared and mounted for permanent viewing. Positive controls were performed with the use of normal gastric tissue and a known PTEN positive case.

In our study, we detected the level of miR-718 as well as the expression of PTEN in gastric tumor tissues, and we analyzed their correlation with the survival rate. With experiments in vitro, we further detected whether miR-718 targeted PTEN directly in gastric cancer cells, and we explored the influence of miR-718 on the proliferation and invasion of gastric cancer.

Background

Gastric cancer is the fourth most common cancer in frequency and the third leading cause of cancer-related deaths worldwide, causing 723,100 deaths in 2012 [1]. Although the surgical equipment and adjuvant therapies have shown remarkably developed over the years, the overall 5-year survival rate of gastric cancer is still unfavorable, remaining lower than 30%, because most patients with gastric cancer are diagnosed at an advanced stage and the recurrence rate is very high [2,3]. In recent years, many predictive and prognostic biomarkers of gastric cancer have been discovered, resulting in the application of new drugs like Herceptin for use as the adjuvant therapy. However, many gastric cancer patients are insensitive to chemotherapy and normally have a lower survival rate than average. Thus, further identification of risk factors and exploration for new drug targets are still urgently needed to control the prevalence and recurrence of gastric cancer.

MicroRNAs (miRNAs) are short (~22 nucleotide) non-protein-coding RNAs which regulate gene expression post-transcriptionally by translational repression or transcriptional degradation [4]. MiRNAs are involved in many cellular processes including apoptosis, differentiation, and proliferation. Ectopic miRNA levels are also observed in several diseases, especially in tumors [5]. Because of tissue-specific expression, miRNAs have great potential as cancer biomarkers, which has been demonstrated in numerous studies [6,7]. The aberrant expression of miRNAs in neoplasms has been observed, and the functions of miRNAs in cell lines has been verified by previous studies [8,9]. This feature of miRNAs as biomarkers could help guide individualized therapy for cancer patients. In gastric cancer, many miRNAs have been demonstrated to be correlated to the progression and prognosis of gastric cancer, including miR-21, miR-22, miR-18a, and miR-222 [10,11].
The score for the positive cell percentage was defined as: 0 for no positively stained cells, 1 for ≤25% positively stained cells, 2 for 25–50% positively stained cells, 3 for 50–75% positively stained cells, and 4 for more than 75% positively stained cells. The final IHC score was the product of the score for staining intensity multiplied by the score for the positive cell percentage, ranging from 0 to 12. The cohort was divided into PTEN high-expression and low-expression group according the cutoff of IHC score, which was set as the point with the highest specificity and sensitivity in the receiver operating characteristic curve (ROC) curve.

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

Quantitative real-time PCR (qRT-PCR) was used to detect the RNA levels of miR-718 and PTEN as previously reported [22]. Total RNA was first extracted from gastric tissues or adjacent tissues with the agent TRizol (Invitrogen, Carlsbad, USA) according to the manual, and dissolved in 10 μL DEPC-treated water. For the detection of PTEN mRNA, the complementary DNA (cDNA) synthesis and quantitative PCR were conducted with SYBR Green Master Mix and StepOnePlus system (Applied Biosystem, Waltham, MA, USA) according to the manual. The level of GAPDH was set as the internal control for PTEN mRNA detection. The sequences of primers for qRT-PCR were as follows: PTEN: forward 5'-GCTATGGAATTCTGACGAAA-3’, reverse 5'-GGCGGTGATCATAATGTCTTCTA-3’; GAPDH: forward 5'-GGATTGTCGTATGGG-3’, reverse 5'-GTGGCTGGGGCTCTACCTC-3’. For the detection of mature miR-718 levels, RNA was reverse-transcribed with miRNA-specific primers and a TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). The small noncoding RNA U6 was used as the internal control for miR-718. The ROC curve was used to set the cutoff of miR-718 to divide the cohort into high-level or low-level miR-718 group.

**Cells and reagents**

Gastric adenocarcinoma cell line MKN-7 was purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, and 100 U/mL penicillin and 100 μg/mL streptomycin (Thermo Fisher Scientific Waltham, MA, USA) in 5% CO₂ resuscitation. All antibodies without special instruction were purchased from Cell Signaling Technology (MA, USA). All reagents without special instruction were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, USA).

**Western blotting**

Cells were scraped with phosphate buffered saline and lysed with ice-cold RIPA lysis buffer (Beyotime Biotecnology, Shanghai, China) supplemented with protease inhibitor cocktail (Roche, Welwyn Garden City, UK). The lysates were centrifuged at 10 000×g for 15 minutes at 4°C and the precipitate was discarded. Equal amount protein (about 10 μg) was electrophoresed in sodium dodecyl sulfate-polyacrylamide gel and then the proteins in gel were transferred to a polyvinylidene difluoride membrane (Merck Millipore, Kenilworth, USA). The primary antibodies including anti-PTEN, anti-AKT, anti-p-Ser473-AKT and anti-β-actin, as well as the corresponding secondary antibodies were used to incubate the membrane, respectively. The bands were finally visualized with the use of an ECL substrate kit (Thermo Scientific, Waltham, MA, USA).

**MTT assay**

MTT assay was used to evaluate the proliferation of gastric adenocarcinoma cells as described be a previous study [23]. Briefly, cells with or without miR-718 transfection were passaged into 96-well plates at a density about 4000 cells per well. After starvation for 6 hours, the test subgroups had added 100 ng/mL epidermal growth factor (EGF), while the control group instead had added normal medium. Finally, 10 μL MTT at the concentration of 5 mg/mL was added to terminate the reaction and 100 μL DMSO was used to dissolve the crystal. The optical density (OD) at 570 nm was measured using a spectrophotometer (Molecular Devices Company, USA). The proliferation ratio of other groups was the ratio of OD570 to the baseline.

**Matrigel invasion assay**

The invasion ability of gastric cancer cells was evaluated by the Matrigel Transwell assay [24]. MKN7 cells about 10³ with or without miR-718 transfection were seeded into 8-μm-core Matrigel precoated Transwell chamber (BD Biosciences Company, Franklin Lakes, NJ, USA) and incubated for 24 hours with 10% fetal bovine serum and 100 ng/mL EGF as chemo-attractants in the lower compartment. The invaded cells in the lower surface were stained with crystal violet. The presented data was the average of triplicate experiments.

**Statistical analysis**

All data in the study were analyzed with software SPSS 21 (SPSS Inc., Chicago, IL, USA). The correlation between PTEN expression and clinicopathological factors was analyzed by χ² test. Student’s t-test was used to analyze the differences in demographic factors between cases and controls. ROC curve was used to determine the cutoff of miR-718 and IHC score of PTEN. The overall survival curve was displayed by Kaplan-Meier
method and the statistical significance between subgroups was compared by the log-rank test. The independent prognostic factors were identified with the Cox regression proportional hazards model. A value of $P<0.05$ was considered statistically significant.

**Results**

**Level of PTEN and miR-718 in gastric cancer and adjacent tissues**

The level of PTEN in gastric cancer was detected by IHC and the miR-718 level was investigated by qRT-PCR. With the cutoff of IHC score and qRT-PCR score respectively, our cohort was divided into high- and low-expression PTEN subgroups or high- and low-level miR-718 subgroups. In our study, the percentage of high-expression PTEN and high-level miR-718 were 63.1% (89 out of 141) and 43.3% (61 out of 141), respectively. The expression of PTEN was mainly observed in the cell cytoplasm in our experiments (Figure 1A, 1B).

Besides the detection of PTEN expression with IHC, we detected the mRNA level of PTEN in a prospective cohort of 12 fresh pairs of gastric cancers and their adjacent tissues with qRT-PCR to quantify the correlation between PTEN and miR-718. The data for the prospective cohort of 141 cases and the prospective cohort with 12 patients are displayed in Supplementary Table 1. In our study, the PTEN mRNA in adjacent tissues was significantly higher compared with that in gastric cancers, suggesting a role for PTEN as a tumor suppressor (Figure 1C). On the contrary, miR-718 levels in adjacent tissues were remarkably lower than that in gastric cancers. This indicated that miR-718 may play an oncogenic role in gastric cancer tumor genesis and progression (Figure 1D).

**Correlation between PTEN, miR-718 and clinicopathological features**

After dividing the cohort into high- and low-expression PTEN subgroups and high- and low-level miR-718 subgroups, we further analyzed the correlations between expression of PTEN, level of miR-718, and other clinicopathological factors including patients’ age, sex, tumor size, tumor infiltration (T stage), lymphatic invasion (N stage), metastasis status (M stage), TNM stage, and tumor cell differentiation (Table 1). With $\chi^2$ test, we demonstrated that the expression of PTEN was significantly associated with miR-718 level. Patients with higher level of miR-718 appeared to have lower expression of PTEN, indicating that miR-718 could inhibit PTEN expression in gastric cancer. No other substantial correlations between PTEN, miR-718, with clinicopathological factors were observed in our study.

**Prognostic value of PTEN and miR-718**

PTEN has been confirmed as a tumor suppressor in gastric cancer in previous studies. In our study, we verified the prognostic significance of PTEN and evaluated the prognostic value of miR-718 simultaneously. The correlation between PTEN, miR-718, and the 5-year overall survival time was analyzed by the univariate analysis using Kaplan-Meier method (Table 2). The T stage, N stage, M stage, TNM stage and tumor differentiation, were all proven to be significantly associated with the 5-year overall survival rate in our study, which was similar to previous study finding (Figure 2A–2E). In our study, PTEN was also identified as a tumor suppressor of gastric cancer. High expression of PTEN led to the favorable prognosis of patients with gastric cancer ($P=0.005$) (Figure 2F). On the contrary, high level of miR-718 was significantly associated with the unfavorable prognosis ($P=0.008$) (Figure 2G).

The multivariate analysis was further performed to identify the independent prognostic factors in the survival of patients with gastric cancer (Table 3). All factors confirmed to be related to the survival rate were enrolled into the Cox-regression hazard model, including the T stage, N stage, M stage, tumor differentiation, PTEN expression and miR-718 level. The TNM stage was naturally excluded because of the known interaction with T stage, N stage and M stage. In our test, all enrolled factors were proven to be the independent prognostic factors of gastric cancer. Although PTEN expression and miR-718 were demonstrated to be significantly correlated, they were both independent prognostic factors of gastric cancer. High expression of PTEN ($P=0.004$, hazard ratio (HR)=0.41, 95% CI=0.22–0.75) alone could predict the favorable prognosis, while the high level of miR-718 ($P=0.018$, HR=2.14, 95% CI=1.14–3.99) was a high-risk biomarker of poorer prognosis to patients with gastric cancer.

**miR-718 could promote gastric cell proliferation and invasion by targeting PTEN**

In the clinical analyses, we observed that a high level of miR-718 was significantly associated with low PTEN expression in gastric cancer tissues, so we further performed function assays to evaluate the role of miRNA in tumor progression. PTEN is a well-known suppressor to PI3K-AKT signaling pathway, therefore, we further explored the effect of miR-718 on regulating PI3K-AKT signaling (Figure 3A). As a generally acknowledged stimulator of PI3K-AKT signaling, EGFR was applied in our study to activate PI3K signaling. In our study, miR-718 could substantially decrease the expression of PTEN, thus increasing the phosphorylation of Ser473 of AKT under stimulation of EGFR. These results suggested that miR-718 could facilitate the activation of PI3K-AKT signaling by degrading PTEN. To detect the influence of miR-718 on tumor cell function, we...
performed MTT assay and Transwell assay to evaluate the proliferation and invasion of gastric cancer cell. In MTT assay, MKN7 cells with miR-718 treatment exhibited an accelerated proliferation compared to the cells without miR-718 treatment (Figure 3B). Similar results were also observed in Transwell assay. Cells with miR-718 treatment had more aggressive ability of invasion compared with those without miR-718 treatment (Figure 3C). These results indicated that miR-718...
### Table 1. Correlations between clinicopathologic factors and PTEN or miR-718.

| Characters                        | PTEN       |          | miR-718    |          |
|----------------------------------|------------|----------|------------|----------|
|                                  | Number     | Low (52) | High (89)  | p*       | Low (80) | High (61)  | p*       |
| **Sex**                          |            |          |            |          |          |            |          |
| Male                             | 102        | 37       | 65         | 0.847    | 61       | 41         | 0.258    |
| Female                           | 39         | 15       | 24         |          | 19       | 20         |          |
| **Age**                          |            |          |            |          |          |            |          |
| <60                              | 59         | 24       | 35         | 0.481    | 30       | 29         | 0.301    |
| ≥60                              | 82         | 28       | 54         |          | 50       | 32         |          |
| **Tumor diameter (cm)**          |            |          |            |          |          |            |          |
| ≤5                               | 62         | 24       | 38         | 0.727    | 32       | 30         | 0.307    |
| >5                               | 79         | 28       | 51         |          | 48       | 31         |          |
| **Differentiation**              |            |          |            |          |          |            |          |
| Well+moderate                    | 78         | 33       | 45         | 0.162    | 41       | 37         | 0.307    |
| Poor                             | 63         | 19       | 44         |          | 39       | 24         |          |
| **Tumor invasion**               |            |          |            |          |          |            |          |
| T1+T2                            | 37         | 10       | 27         | 0.169    | 25       | 12         | 0.176    |
| T3+T4                            | 104        | 42       | 62         |          | 55       | 49         |          |
| **Lymph node metastasis**        |            |          |            |          |          |            |          |
| No (N0)                          | 54         | 20       | 34         | 1.000    | 36       | 18         | 0.080    |
| Yes (N1/2/3)                     | 87         | 32       | 55         |          | 44       | 43         |          |
| **Distant metastasis**           |            |          |            |          |          |            |          |
| M0                               | 130        | 49       | 81         | 0.746    | 72       | 58         | 0.350    |
| M1                               | 11         | 3        | 8          |          | 8        | 3          |          |
| **TNM stage**                    |            |          |            |          |          |            |          |
| I–II                             | 64         | 22       | 42         | 0.603    | 37       | 27         | 0.865    |
| III–IV                           | 77         | 30       | 47         |          | 43       | 34         |          |
| **PTEN**                         |            |          |            |          |          |            |          |
| Low                              | 52         | 21       | 31         |          | 21       | 31         | 0.005    |
| High                             | 89         |          |            |          |          |            |          |
| **miR-718**                      |            |          |            |          |          |            |          |
| Low                              | 80         | 21       | 59         | 0.005    |          |            |          |
| High                             | 61         | 31       | 30         |          |          |            |          |

* Means calculated by χ² test. PTEN – phosphatase and tensin homolog.
in gastric cancer cells could promote the proliferation and invasion processes.

**Discussion**

Gastric cancer is still a major public health problem and a medical financial burden worldwide. The discovery of new biomarkers would help broaden the insights of gastric cancer progression and help find effective drug targets. Elucidation of mechanisms underlying how human epidermal growth factor receptor-2 (HER2) led to poor prognosis of gastric cancer directly resulted in the application of trastuzumab in the treatment of gastric cancer and improved patient survival time. Similarly, the discovery of miRNAs could expand the understanding of carcinogenesis and progression of gastric cancer. Recently, many miRNAs have been demonstrated to be associated with the progression and outcome of gastric cancer, including miR-206, miR-421, and miR-22 [25–27]. Chang et al. revealed that many potential miRNAs were associated with the outcome of gastric cancer patients following chemotherapy using a high-throughput miRNA microarray analysis [28]. Accumulating evidence shows that miRNAs are effectively predictive and prognostic biomarkers in gastric cancer [29,30]. MiRNAs have the potency to predict prognosis and guide the treatment. Some miRNAs could help predict sensitivity to chemotherapy and help find effective chemo-drugs, and some miRNAs could help predict the prognosis of patients [31]. Exploration of more prognostic miRNAs in gastric cancer could help distinguish the patients with higher risk for more potential adjuvant therapy, thus defining appropriate treatment options for patients.

As a dual-specificity protein phosphatase, PTEN is a well-acknowledged tumor suppressor in many kinds of cancers [32,33]. The tumor suppressor role of PTEN was also elucidated in several previous studies. PTEN was involved in many processes in gastric cancer, including tumor genesis, progression, chemotherapeutic drug sensitivity, and prognosis [12,34–36]. Since PTEN could suppress PI3K-AKT signaling, the loss of PTEN function resulting from gene mutation or deletion, would increase phosphatidylinositol 3-phosphate (PIP3), which is the product of the PI3K pathway [33]. The accumulation of PIP3 could activate a signaling cascade leading to the activation and phosphorylation of AKT and the downstream proteins, by which PTEN is involved in many cellular processes such as cell survival, proliferation, and invasion [37]. The mRNA of PTEN is featured with its large 3'UTR (about 3.3kbp), which could be targeted by several miRNAs including miR-21, miR-214, miR-216a, miR-217, and miR-26a [38–42]. Recently, miR-718 was reported to repress the pro-inflammatory cytokine production through
targeting PTEN, and it has been suggested that miR-718 could function as an oncogene [14]. In our study, we demonstrated that miR-718 could promote gastric cell proliferation and invasion via targeting PTEN mRNA, and that high level miR-718 was an independent prognostic risk factor for a poorer prognosis of gastric cancer. These results add to the study on gastric cancer progression and treatment and could help stratify patients more precisely for individual treatment.
Figure 3. miR-718 could activate PI3K/Akt signaling by targeting PTEN. (A) The transcription of PTEN in gastric cell line was suppressed by miR-718. The normalized firefly luciferase activity of PTEN was detected 48 hours after the transfection with the PTEN-3’UTR-Luc reporter construct with the absence of miR-718 expression vector or the scrambled control miRNA vector in gastric cancer cell MKN7. Data were from 3 independent experiments and displayed by the mean ±SEM. (B) PI3K/Akt signaling in MKN7 could be activated by miR-718. The effect of miR-718 on Akt signaling of MKN7 cells was evaluated by western blotting. EGF at 100 ng/mL was used to incubate the cells for 10 minutes, 48 hours after transfection of expression vector of miR-718 or scrambled control miRNA. Cells were then lysed, and western blotting was performed to detect the levels of PTEN, phosphor-Ser473-Akt, total Akt, and β-actin. (C) miR-718 could accelerate the proliferation of MKN7. The MTT assay was used to detect the proliferation index of MKN7 48 hours after transfection of expression vector of miR-718 or scrambled control miRNA. Data were from 3 independent experiments and displayed by the mean ±SEM. (E) Representative images of invaded cells in D. Image 1, 2, 3, 4 represent the groups with/without EGF stimulation or with/without miR-718 transfection.

Table 3. Multivariate analysis.

| Characters          | HR  | 95%CI         | P*  |
|---------------------|-----|---------------|-----|
| Tumor invasion      |     |               |     |
| T1+T2               | 1   |               |     |
| T3+T4               | 2.79| 1.23–6.32     | 0.014|
| Lymph node invasion |     |               |     |
| No (NO)             | 1   |               |     |
| Yes (N1/2/3)        | 1.94| 1.06–3.55     | 0.031|
| Distant metastasis  |     |               |     |
| M0                  | 5.18| 2.04–13.14    | 0.001|
| M1                  |     |               |     |

* Means calculated by Cox-regression model. PTEN – phosphatase and tensin homolog.

Conclusions

We demonstrated that expression of PTEN and miR-718 were significantly correlated in patients with gastric cancer. Low expression of PTEN and high level of miR-718 were notably associated with a lower 5-year overall survival rate. Both PTEN and miR-718 were identified as prognostic factors of gastric cancer. With experiments in vitro, we proved that miR-718 could increase PI3K/Akt signaling by directly downregulating PTEN, thus promoting the proliferation and invasion of gastric cancer cells.
Conflicts of interest

None.

Supplementary Table

Supplementary Table 1. Basic information of the retrospective cohort and prospective cohort.

| Characters                  | Retrospective cohort | Prospective cohort |
|-----------------------------|----------------------|--------------------|
|                             | Number   | Percentage | Number   | Percentage |
| Sex                         |          |            |          |            |
| Male                        | 102      | 72.3%      | 9        | 75.0%      |
| Female                      | 39       | 27.7%      | 3        | 25.0%      |
| Age                         |          |            |          |            |
| <60                         | 59       | 41.8%      | 3        | 25.0%      |
| ≥60                         | 82       | 58.2%      | 9        | 75.0%      |
| Tumor diameter (cm)         |          |            |          |            |
| ≤5                          | 62       | 44.0%      | 5        | 41.7%      |
| >5                          | 79       | 56.0%      | 7        | 58.3%      |
| Differentiation             |          |            |          |            |
| Poor                        | 78       | 55.3%      | 6        | 50.0%      |
| Well+moderate               | 63       | 44.7%      | 6        | 50.0%      |
| Tumor invasion              |          |            |          |            |
| T1+T2                       | 37       | 26.2%      | 5        | 41.7%      |
| T3+T4                       | 104      | 73.8%      | 7        | 58.3%      |
| Lymph node metastasis       |          |            |          |            |
| No (N0)                     | 54       | 38.3%      | 6        | 50.0%      |
| Yes (N1/2/3)                | 87       | 61.7%      | 6        | 50.0%      |
| Distant metastasis          |          |            |          |            |
| M0                          | 130      | 92.2%      | 11       | 91.7%      |
| M1                          | 11       | 7.8%       | 1        | 8.3%       |
| TNM stage                   |          |            |          |            |
| I–II                        | 64       | 45.4%      | 5        | 41.7%      |
| III–IV                      | 77       | 54.6%      | 7        | 58.3%      |

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