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1 # Silencing of lncRNA NEAT1 inhibits esophageal squamous cell
2 carcinoma proliferation, migration, and invasion via regulation
3 of the miR-1299/MMP2 axis

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9 **Abstract:**
10 Esophageal squamous cell carcinoma (ESCC) is the most prevalent form of esophageal cancer worldwide.
11 Considerable evidence has verified that abnormal expression of lncRNAs can effectively influence the
12 progression of various malignant tumors. However, the regulatory mechanisms of lncRNAs underlying
13 ESCC development and progression remain poorly defined. Here, we investigated the role of lncRNA
14 nuclear-enriched abundant transcript 1 (NEAT1) in ESCC via regulating microRNA 1299 (miR-1299) and
15 matrix metalloproteinase 2 (MMP2). A total of 32 ESCC tissue samples were obtained from the First
16 Affiliated Hospital of Zhengzhou University. The mRNA levels of lncRNA NEAT1, miR-1299, and
17 MMP2 mRNA were measured via quantitative real-time PCR. Interactions among miR-1299, lncRNA
18 NEAT1, and MMP2 mRNA in EC9706 cells were confirmed by dual-luciferase reporter assays and RNA
19 immunoprecipitation (RIP) assays. The proliferation and migration/invasion of ESCC cells were verified by
20 CCK-8 and transwell assays, respectively. lncRNA NEAT1 was up-regulated in ESCC tissues and cells.
21 lncRNA NEAT1 silencing inhibited migration, invasion, and proliferation of ESCC cells. Furthermore,
22 lncRNA NEAT1 sponged and negatively regulated miR-1299, thus giving rise to increased expression of
23 MMP2. Moreover, miR-1299 inhibitors and MMP2 rescued the invasion of ESCC cells following silencing
24 of lncRNA NEAT1. lncRNA NEAT1 was overexpressed in ESCC tissues and cells. Silencing of lncRNA
25 NEAT1 inhibited ESCC proliferation, migration, and invasion via reducing competitive binding of lncRNA

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26 NEAT1 with miR-1299 and enhancing miR-1299-targeted suppression of MMP2. Taken together, our
27 findings suggest that lncRNA NEAT1 is a potential target for ESCC therapy and rehabilitation.

28 **Keywords:** lncRNA NEAT1; Esophageal squamous cell carcinoma; MiR-1299; CeRNA; MMP2

29 Esophageal squamous cell carcinoma (ESCC) is the most prevalent form of esophageal cancer worldwide
30 and exhibits a high incidence rate and fatality rate worldwide, especially in East Asia, East Africa, South
31 Africa, and Southern Europe[1, 2]. Clinically, despite enormous advances having been made in
32 preoperative radiotherapy, chemotherapy, and surgery in the past several years, the chance of survival for
33 ESCC patients has been improved but the five-year survival rate has remained at only 15%–20%, with a
34 median survival time of approximately 1.5 years[3]. Moreover, because the early symptoms are not
35 obvious, the overwhelming majority of ESCC patients are diagnosed at an advanced stage when tumors
36 cannot be thoroughly surgically removed[4]. Poor prognosis is a direct result of neoplasm metastasis,
37 radiotherapy resistance, and the high recurrence rate of ESCC.[5] Thus, identifying more exploitable
38 molecular markers for early and metaphase diagnosis, as well as elucidating underlying regulatory
39 mechanisms of ESCC, are urgently needed for ESCC patients to improve prognosis and survival.

40 Long non-coding RNAs (lncRNAs), each of which are over 200 nt in length, are transcribed by RNA
41 polymerase and are produced by splicing and modification of small nuclear or mature RNAs, but do not
42 have any capacity for encoding proteins[6]. Previous evidence has indicated that lncRNAs may regulate
43 numerous biological processes such as tumor growth and metastasis, including those involved in ESCC[7].
44 Moreover, lncRNAs play roles as signals, scaffolds, guides, decoys, and competing endogenous RNAs
45 (ceRNAs) through interacting with their corresponding target genes[8]. In various cancers, many lncRNAs
46 have been discovered to exhibit abnormal expression levels and to be closely related to tumor progression.
47 For instance, lncRNA ZFAS1 is up-regulated in ESCC and promotes cell proliferation, migration, and
48 invasion in ESCC cell lines by downregulating miR-124 and upregulating STAT3[9]. lncRNA KLF3-AS1
49 is down-regulated in ESCC and inhibits tumor growth and invasion via weakening miR-185-5p-mediated
50 inhibition of KLF3[10]. lncRNA PANDA is upregulated in ESCC and drifts away from NF-YA to promote
51 the expression of NF-YA-E2F1 co-regulated proliferation-promoting genes, as well as to restrict
52 apoptosis[11]. lncRNA HAGLR is highly expressed in esophagus cancer (EC) , and HAGLR depletion
53 leads to cell proliferation, metastasis, invasion, and inhibition of the epithelial-mesenchymal transition

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54 (EMT) by restoring the expression of miR-143-5p and downregulating LAMP3[12]. Additionally, nuclear
55 enriched abundant transcript 1 (NEAT1) is an lncRNA that has been verified to be a carcinogen in diverse
56 malignancies, including colorectal cancer, breast cancer, hepatocellular carcinoma, lung cancer, melanoma
57 cancer, prostate cancer, pancreatic cancer, and ovarian cancer (OC)[13-20].
58 Furthermore, many studies have demonstrated that abnormal expression patterns of miRNAs serve as
59 tumor-inhibiting factors and carcinogens in multiple human malignancies, including ESCC[21]. MiR-1299
60 has been shown to be down-regulated in diverse cancers, including colon cancer, prostate cancer,
61 hepatocellular carcinoma, and ovarian cancer; furthermore, miR-1299 has also been demonstrated to act as
62 a tumor suppressor[22-25]. Additionally, matrix metalloproteinase 2 (MMP2) has been identified as an
63 important gene for invasion and lymph-node metastasis in ESCC, and overexpression of MMP2 protein in
64 ESCC tissues verifies that it is closely associated with esophageal metastasis[26]. According to the gene
65 sequences of these aforementioned molecules matched via bioinformatic analysis and literature review, we
66 hypothesized that lncRNA NEAT1 may influence proliferation, migration, and invasion of ESCC via
67 regulating miR-1299 and MMP2. Hence, in the current study, we investigated the potential molecular
68 mechanisms of lncRNA NEAT1 in regulating miR-1299 and MMP2 in ESCC, with the aim of identifying a
69 novel strategy for ESCC therapy and rehabilitation.

70 Materials and methods

71 **Main experimental materials.** Normal human esophageal epithelial cells (Het-1A) and human ESCC cell
72 lines (KYSE30, KYSE150, and EC9706 cells) were all provided by the Shanghai Cell Bank at the Chinese
73 Academy of Sciences (Shanghai, China). SiRNA lncRNA NEAT1 (si-NEAT1), si-RNA negative controls
74 (si-NCs), an miR-1299 inhibitors, miR-1299 mimics (miR-1299), and microRNA negative controls (miR-
75 NCs) were obtained from RiboBio (Guangzhou, China). Lacking 3'-UTR MMP2 overexpression
76 plasmids (pcDNA-MMP2) were purchased from GenePharma (Shanghai, China).
77 **Patient specimens.** A total of 32 pairs of ESCC tissue and para-cancer tissue samples were obtained from
78 the First Affiliated Hospital of Zhengzhou University. These patients had not received radiotherapy or
79 chemotherapy before surgery. Immediately after surgical excision, these tissue specimens were stored
80 directly in liquid nitrogen until further use. Ethical approval for this study was approved by the Ethics

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81 Committee of Zhengzhou University. All procedures were in accordance with the Declaration of Helsinki
82 and written informed consent was obtained from each patient in advance.
83 **Cell cultures and transfections.** Cells were cultured in RPMI-1640 (Hyclone, Logan, UT, USA)
84 supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) in a humidified
85 atmosphere at 37°C with 5% (v/v) CO_2. Si-NEAT1, si-NCs, miR-1299 mimics, miR-NCs, miR-1299
86 inhibitors, and pcDNA-MMP2 were transfected into KYSE30 and EC9706 cells via Lipofectamine 2000
87 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.
88 **Quantitative real-time PCR (qRT-PCR).** Total RNAs were extracted separately from tissue samples and
89 cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) on the basis of the manufacturer's
90 protocol. Then, lncRNA NEAT1 and MMP2 mRNA were reversed transcribed into cDNA using
91 PrimeScript RT Reagent Kit (Takara, Dalian, China), and miR-1299 was reversed transcribed into cDNA
92 using TaqMan microRNA Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA). Subsequently, RNA
93 levels were measured via quantitative real-time qPCR (qRT-PCR) on a Bio-Rad CFX96 Touch System
94 (Bio-Rad, Hercules, CA, USA). The primers used in this study were synthesized by Tsingke (Tsingke,
95 Beijing, China) and are listed in Table 1.
96 **Cell counting kit-8 (CCK8) assays.** Cell proliferation was measured by Cell Counting Kit-8 assays
97 (Dojindo Molecular Laboratories, Japan). Initially, the transfected KYSE30 and EC9706 cells were seeded
98 in 96-well plates (2×10^3 cells/well) and cultured for several days at 37°C with 5% (v/v) CO_2. Then, 10 μL
99 of CCK-8 solution was added to the cells and incubated for 1 h at 0, 24, 48, and 72 h. Subsequently, the
100 optical density (OD) value of each sample at 450 nm was measured by a microplate reader (ELx800;
101 BioTek, Winooski, VT, USA).
102 **Transwell migration and invasion assays.** Transwell assays were conducted to assess the migration and
103 invasion of ESCC cells. After transfection, KYSE30 and EC9706 cells (1×10^4 cells/well) were suspended
104 in serum-free medium and seeded onto the upper chamber of inserted 8-μm-pore filters coated with
105 Matrigel (BD Biosciences, San Jose, CA, USA) for invasion assays or uncoated for migration assays.
106 RPMI-1640 medium containing 10% FBS was added to the lower chamber. After incubation for 24 h, the
107 cells having no invasive or migratory activities were erased from the upper surface of the chamber with
108 cottons swabs and the invaded and migrated cells on the lower surface of the membrane were fixed with

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109 4% paraformaldehyde for 30 min, after which they were stained with 0.3% crystal violet for 5 min. Then,
110 nine regions were randomly selected and the numbers of cells in these regions were counted with an
111 inverted microscope (Nikon, Tokyo, Japan).
112 **Dual-luciferase reporter assays.** Bioinformatic analyses of miRDB and lncBase v2 were explored to
113 search and obtain three binding sites of lncRNA NEAT1 and miR-1299. Then, pmirGLO plasmids
114 containing three pairs of wild-type and mutant binding sequences were purchased from GeneChem
115 (Shanghai, China), including pmirGLO-Wt1, pmirGLO-Mt1, pmirGLO-Wt2, pmirGLO-Mt2, pmirGLO-
116 Wt3, and pmirGLO-Mt3. Meanwhile, wild-type and mutant MMP2 3′-UTRs were synthesized and
117 recombined into pmirGLO Luciferase vectors (Promega, Madison, WI, USA). Then pmirGLO-
118 Wt/pmirGLO-Mt or miR-1299 mimics/miR-NCs were co-transfected into EC9706/KYSE30 cells. After 48
119 h of transfection, firefly luciferase activities of cells were measured via dual-luciferase reporter assays
120 (Promega, Madison, WI, USA).
121 **RNA immunoprecipitation (RIP) assays.** RIP assays were conducted to explore the relationship between
122 lncRNA NEAT1 and miR-1299 via the Magna RIP RNA-Binding Protein Immunoprecipitation Kit
123 (Millipore, MA, USA). Initially, digested EC9706 cells were harvested and lysed with complete RIP lysis
124 buffer. Afterword, the supernatants of cell lysates were incubated with magnetic beads coated with anti-
125 Ago 2 or control anti-IgG (Millipore, MA, USA) for 4 h at 4°C. Then, the immunoprecipitation complex
126 was washed six times with cold RIP wash buffer. Finally, the RNA levels of extracted lncRNA NEAT1 and
127 miR-1299 were detected by qRT-PCR.
128 **Statistical analysis.** GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) and Statistical SPSS 19.0
129 (IBM, NY, USA) software were used for statistical analysis. All assays were independently repeated three
130 times under the same conditions. Moreover, all data were reported as the mean ± standard deviation (SD).
131 Linear correlation analysis and independent sample t-tests were used for analysis. A $P<0.05$ was
132 considered to indicate a statistically significant difference.
133

134 ## Results

135 **lncRNA NEAT1 is upregulated in ESCC tissues and cell lines.** We measured RNA levels of lncRNA
136 NEAT1, miR-1299, and MMP2 in 32 ESCC tissues, as well as in multiple ESCC cell lines, via qRT-PCR.

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137 The results showed that lncRNA NEAT1 and MMP2 in ESCC tissues were significantly up-regulated
138 compared with those in para-cancer tissues (*P<0.05*) (Figures 1A, 1C). Meanwhile, miR-1299 in ESCC
139 tissues was significantly down-regulated (*P<0.05*) (Figure 1B). Analysis of clinicopathological
140 characteristics indicated that miR-1299 levels in ESCC tissues were not significantly different as a function
141 of gender, age, lymph-node metastasis status, or and TNM stage. In contrast, lncRNA NEAT1 and MMP2
142 in ESCC tissues with lymph-node metastasis were significantly higher than those without lymph-node
143 metastasis (*P<0.05*); additionally, lncRNA NEAT1 and MMP2 in ESCC tissues at the TNM-III stage were
144 significantly higher than those at stage I or stage II (*P<0.05*) (Table 2). Furthermore, lncRNA NEAT1 and
145 MMP2 in three ESCC cell lines were significantly upregulated compared with those in normal human
146 esophageal epithelial cells (Het-1A) (*P<0.05*) (Figures 1D, 1F). Conversely, miR-1299 levels in three
147 ESCC cell lines were significantly downregulated compared with those in Het-1A cells (*P<0.05*) (Figure
148 1E). These results suggested that lncRNA NEAT1 and MMP2 were overexpressed in ESCC tissues and
149 cells, whereas miR-1299 exhibited low expression. Collectively, these findings suggest that up-regulation
150 of lncRNA NEAT1 was related to the progression of ESCC.
151 **lncRNA NEAT1 silencing impairs ESCC proliferation, invasion, and migration.** KYSE30 and EC9706
152 cells were used to further investigate the function of lncRNA NEAT1. First, we verified the knockdown
153 efficiency of lncRNA NEAT1 in KYSE30 and EC9706 cells (Figure 2A). Next, CCK8 assays were
154 performed in KYSE30 and EC9706 cells. The results indicated that knockdown lncRNA NEAT1
155 significantly impaired cellular proliferation in KYSE30 and EC9706 cells (Figures 2B, 2C). Additionally,
156 transwell assays demonstrated that lncRNA NEAT1 knockdown also attenuated invasion and migration of
157 KYSE30 and EC9706 cells (Figures 2D, 2E). Therefore, these results validated that silencing of lncRNA
158 NEAT1 inhibited proliferation, invasion, and migration of ESCC.
159 **lncRNA NEAT1 sponges miR-1299 in ESCC cells.** We next explored potential targets of lncRNA
160 NEAT1 via bioinformatic analysis (LncBase v2 and miRDB), among which miR-1299 was found to have
161 the highest target score (Figure 3A). Then, to verify the binding level of lncRNA NEAT1 with miR-1299,
162 dual-luciferase reporter assays and RIP assays were executed with EC9706 cells. The data indicated that
163 the activity of luciferase was inhibited by miR-1299 in pmirGLO-WT1,2,3 containing three binding sites
164 (Figures 3B–3D). In addition, lncRNA NEAT1 and miR-1299 in anti-Ago2-immunoprecipitated complexes

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165 were significantly enriched compared with those in control anti-IgG-immunoprecipitated complexes
166 (*P<0.05*) (Figure 3E), verifying that lncRNA NEAT1 and miR-1299 could simultaneously bind to anti-
167 Ago2. The above data indicated that lncRNA NEAT1 interacted directly with miR-1299. Subsequently, to
168 better elucidate the interaction of lncRNA NEAT1 and miR-1299 in ESCC, qRT-PCR assays were
169 executed with KYSE30 and EC9706 cells. The data revealed that silencing of lncRNA NEAT1 induced up-
170 regulation of miR-1299 in KYSE30 and EC9706 cells (Figure 3F). Additionally, we found that there was a
171 significantly negative correlation between lncRNA NEAT1 and miR-1299 levels in ESCC tissues (Figure
172 3G). Taken together, these findings demonstrated that lncRNA NEAT1 sponges and negatively regulates
173 miR-1299 as a ceRNA in ESCC cells.

174 **MiR-1299 suppresses invasion and migration by targeting MMP2 in ESCC.** First, we confirmed that
175 increased miR-1299 negatively regulated the expression of MMP2 at the mRNA level (Figure 4A). Second,
176 we substantiated the suppressive effect of miR-1299 acting on invasion and migration in KYSE30 and
177 EC9706 cells. Moreover, we discovered that the suppressive effect of miR-1299 could be reversed by
178 pcDNA-MMP2 (Figures 4B, 4C). Finally, by performing dual-luciferase reporter assays, we validated that
179 miR-1299 targeted MMP2 with the predicted binding sites (Figures 4D–4F). In summary, these results
180 demonstrated that miR-1299 directly targeted MMP2 and weakened its mediated invasion and migration in
181 KYSE30 and EC9706 cells.

182 **lncRNA NEAT1 silencing suppresses MMP2-mediated invasion by reducing the competitively-**
183 **binding miR-1299 in ESCC cells.** Next, we investigated whether lncRNA NEAT1 regulated ESCC
184 invasion by miR-1299/MMP2. Transwell rescue assays were performed with KYSE30 and EC9706 cells.
185 As shown in Figures 5A and 5B, silencing of lncRNA NEAT1 suppressed invasion in KYSE30 and
186 EC9706 cells. Meanwhile, miR-1299 inhibitors and pcDNA-MMP2 promoted invasion. The data validated
187 that miR-1299 inhibitors and pcDNA-MMP2 significantly rescued the invasion of KYSE30 and EC9706
188 cells following silencing of lncRNA NEAT1 ($P<0.05$). Thus, our findings elucidated that silencing of
189 lncRNA NEAT1 reduced the competitively-binding miR-1299 to inhibit MMP2-mediated invasion in
190 ESCC cells.
191

192 Discussion

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193 Many studies have demonstrated that lncRNAs perform crucial roles in the progression of malignant
194 tumors and may also represent novel targets for oncotherapy[27]. Therefore, the current study was
195 performed to investigate the function of lncRNA NEAT1 in ESCC. We found that lncRNA NEAT1 was
196 highly expressed in ESCC tissue samples and served as a ceRNA via sponging miR-1299 to upregulate
197 MMP2, which ultimately promoted the progression of ESCC.
198 Initially, in our present study, we discovered the lncRNA NEAT1 was upregulated in 32 ESCC tissue
199 samples and in three different cell lines. Furthermore, the expression level of lncRNA NEAT1 was closely
200 associated with lymph-node metastasis and clinical stage. Additionally, silencing of lncRNA NEAT1
201 suppressed cell proliferation, invasion, and migration, consistent with previous research.[28] Moreover, we
202 found that lncRNA NEAT1 sponged and inhibited miR-1299. Recently, lncRNAs have been confirmed to
203 serve as ceRNAs via interacting with miRNA seed sequences[9, 10, 12]. For instance, lncRNA MEG3
204 participates in the inhibition of cell proliferation and invasion by serving as a ceRNA to regulate the
205 expression of FOXO1 and E-cadherin via competitively binding miR-9 in ESCC[29]. In addition, lncRNA
206 HOTAIR promotes the progression of EMT by acting as a ceRNA to regulate ZEB1 expression through the
207 sponging of miR-130a-5p in ESCC[30]. Therefore, we hypothesized that lncRNA NEAT1 might
208 competitively bind specific miRNAs to exert the inhibitory effects in ESCC. To test this hypothesis, we
209 searched for potential miRNAs interacting with lncRNA NEAT1 through miRDB and LncBase v2. We
210 ultimately identified that miR-1299 had the highest target score with lncRNA NEAT1 and exhibited
211 binding to three sites simultaneously. Moreover, qRT-PCR assays suggested that the expression of miR-
212 1299 was negatively correlated with that of lncRNA NEAT1 in ESCC cells and tissue samples.
213 Furthermore, based on dual-luciferase reporter assays and RIP assays, we verified that miR-1299 was a
214 direct target of lncRNA NEAT1 and possessed three binding regions. Interestingly, miR-1299 has been
215 confirmed to be a tumor-inhibiting factor[21-25].Collectively, these results demonstrate that lncRNA
216 NEAT1 acted as a ceRNA to enhance cell proliferation, invasion, and migration by sponging miR-1299 in
217 ESCC.
218 Subsequently, we found that miR-1299 overexpression reduced the expression of MMP2 mRNA. Previous
219 studies have shown that miR-1299 inhibits the progression of diverse human tumors by targeting mRNAs
220 for molecular proteins[25, 31]. For example, miR-1299 inhibits the growth, invasion, and metastasis of

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221 prostate cancer via regulating NEK2[22]. Inhibition of miR-1299 expression increases the proliferation,
222 cell-cycle progression, and invasion of HCC cells by promoting CTNND1 expression[32]. In the current
223 study, we found that miR-1299 negatively regulated MMP2 expression and the pcDNA-MMP2 weakened
224 the ability of miR-1299 to inhibit invasion and migration in KYSE30 and EC9706 cells, suggesting that
225 miR-1299 was involved in MMP2-mediated invasion and migration. Furthermore, bioinformatics and dual-
226 luciferase assays confirmed that MMP2 was a direct target of miR-1299 in ESCC cells. Finally, we found
227 that silencing of lncRNA NEAT1 also inhibited MMP2-mediated invasion by reducing the competitively
228 binding miR-1299 in KYSE30 and EC9706 cells. By matching the seed sequences of lncRNA NEAT1 and
229 MMP2 binding with miR-1299, we discovered that lncRNA NEAT1 and MMP2 shared the same base
230 sequences for miR-1299. Moreover, based on transwell rescue assays, si-NEAT1 suppressed invasion,
231 whereas miR-1299 inhibitors and pcDNA-MMP2 markedly reversed the suppressive effect of silencing
232 lncRNA NEAT1 in KYSE30 and EC9706 cells. These results indicate that there is an ESCC-specific
233 ceRNA network based on expression profiles of lncRNAs, miRNAs, and mRNAs[33]. Furthermore, similar
234 ceRNA networks have been elucidated in numerous previous studies[10,12,14,16]. Collectively, these
235 results strongly suggest that the supressed effect of lncRNA NEAT1 silencing on MMP2 in our present
236 study was accomplished via the miR-1299 pathway.
237 In conclusion, we found that lncRNA NEAT1 was overexpressed in ESCC tissues and cells, and was
238 associated with lymph-node metastasis and TNM stage.Silencing of lncRNA NEAT1 inhibited ESCC
239 proliferation, invasion, and migration via reducing the competitively-binding miR-1299 and enhancing
240 miR-1299-targeted suppression of MMP2. Our present findings provide novel evidence that lncRNAs
241 function as ceRNAs of miRNAs, and that lncRNA NEAT1 may represent a promising target for ESCC
242 therapy and rehabilitation.
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244
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340

341 **Figure Legends**

342 **Figure 1.** lncRNA NEAT1 is upregulated in ESCC tissues and cells. A) The expression levels of lncRNA
343 NEAT1 in 32 ESCC tissues and their para-carcinoma tissues were analyzed by qRT-PCR. B) The
344 expression levels of miR-1299 in 32 ESCC tissues and their para-carcinoma tissues were analyzed by qRT-
345 PCR. C) The expression levels of MMP2 mRNA in 32 ESCC tissues and their para-carcinoma tissues were
346 analyzed by qRT-PCR. D) Relative expression levels of lncRNA NEAT1 in Het-1A cells and ESCC cells
347 were measured by qRT-PCR. E) Relative expression levels of miR-1299 in Het-1A cells and ESCC cells
348 were measured by qRT-PCR. F) Relative expression levels of MMP2 mRNA in Het-1A cells and ESCC
349 cells were measured by qRT-PCR. $^{*}P<0.05$

350

351 **Figure 2.** lncRNA NEAT1 silencing impairs ESCC proliferation, invasion, and migration. A) Relative
352 expression levels of lncRNA NEAT1 in KYSE30 and EC9706 cells transfected with si-NC, or si-NEAT1.
353 B) CCK8 assays revealed that si-NEAT1 impaired the proliferation in KYSE30 cells. C) CCK8 assays
354 revealed that si-NEAT1 impaired the proliferation in EC9706 cells. D) Transwell assays of cell invasion
355 revealed that lncRNA NEAT1 knockdown impaired invasion of KYSE30 and EC9706 cells. E) Transwell
356 assays of cell migration revealed that lncRNA NEAT1 knockdown impaired migration of KYSE30 and

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357 EC9706 cells. *P<0.05

358

359 **Figure 3.** lncRNA NEAT1 sponges and negatively regulates miR-1299 in ESCC cells. A) Chart showing
360 three inferred binding sites of lncRNA NEAT1 with miR-1299. The regions marked with an underscore
361 were the mutated sites in lncRNA NEAT1. B) Dual-luciferase reporter assays were executed with EC9706
362 cells and showed that the activity of luciferase was inhibited by miR-1299 in pmirGLO-Wt1. C) Dual-
363 luciferase reporter assays showed that the activity of luciferase was inhibited by miR-1299 in pmirGLO-
364 Wt2. D) Dual-luciferase reporter assays showed that the activity of luciferase was inhibited by miR-1299 in
365 pmirGLO-Wt3. E) RIP assays were executed with EC9706 cells and showed that lncRNA NEAT1 and
366 miR-1299 in anti-Ago2-immunoprecipitations were enhanced compared with those in control anti-IgG-
367 immunoprecipitations. F) Silencing of lncRNA NEAT1 promoted miR-1299 expression in KYSE30 and
368 EC9706 cells. G) A significant negative linear correlation was found between lncRNA NEAT1 levels and
369 miR-1299 levels in ESCC tissues. *P<0.05

370

371 **Figure 4.** MiR-1299 weakens invasion and migration through targeting MMP2 in ESCC. A) The
372 expression levels of MMP2 mRNA in KYSE30 and EC9706 cells transfected with miR-NC and miR-1299
373 mimics were analyzed by qRT-PCR. B) Transwell assays of cell invasion in KYSE30 and EC9706 cells
374 transfected with miR-NC, miR-1299 mimics, pcDNA-MMP2, and miR-1299 mimics+pcDNA-MMP2. C)
375 Transwell assays of cell migration in KYSE30 and EC9706 cells transfected with miR-NC, miR-1299
376 mimics, pcDNA-MMP2, and miR-1299 mimics+pcDNA-MMP2. D) Chart of predicted miR-1299 binding
377 sites of MMP2 3'-UTR. E) MiR-1299 inhibited the activity of luciferase in KYSE30 cells co-transfected
378 with pmirGLO-Wt-MMP2 3'-UTR. F) MiR-1299 inhibited the activity of luciferase in EC9706 cells co-
379 transfected with pmirGLO-Wt-MMP2 3'-UTR. *P<0.05

380

381 **Figure 5.** lncRNA NEAT1 silencing suppresses MMP2-mediated invasion by reducing the competitively-
382 binding miR-1299 in ESCC cells. A) Transwell rescue assays of cell invasion in KYSE30 cells transfected
383 with si-NEAT1, miR-1299 inhibitor, si-NEAT1+miR-1299 inhibitor, pcDNA-MMP2, and si-
384 NEAT1+pcDNA-MMP2. B) Transwell rescue assays of cell invasion in EC9706 cells transfected with si-

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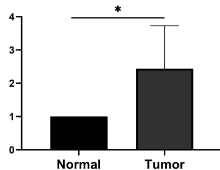
385 NEAT1, miR-1299 inhibitor, si-NEAT1+miR-1299 inhibitor, pcDNA-MMP2, and si-NEAT1+pcDNA-
386 MMP2. *P<0.05
387
388 **Table 1.** Primer sequences used for qRT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
lncRNA NEAT1	TGGCTAGCTCAGGGCTTCAG	TCTCCTTGCCAAGCTTCCTTC
miR-1299	CCCTAACGGTTCTGGAATTCTGT	TATGGTTGTTCACGACTCCTTCAC
GADPH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
MMP2	GGAACGCCGATGGGGAGTA	CCTGGAAGCGGAATGGAAA

389
390 **Table 2.** Clinicopathologic characteristics and lncRNA NEAT1, MiR-1299, and MMP2 levels in 32 ESCC
391 samples

Characteristics	lncRNA NEAT1 levels	*P*-value	miR-1299 levels	*P*-value	MMP2 levels	*P*-value
Gender						
Female	2.31±1.35	0.592	0.61±0.32	0.651	2.06±0.46	0.097
Male	2.56±1.26		0.66±0.37		2.33±0.43	
Age						
≤60	2.61±1.19	0.500	0.69±0.38	0.382	2.15±0.43	0.562
>60	2.29±1.39		0.59±0.31		2.25±0.49	
TNM Stage						
I + II	1.97±1.18	0.016*	0.70±0.35	0.233	2.02±0.38	0.008*
III	3.05±1.20		0.55±0.32		2.44±0.45	
Lymph-Node Metastasis						
Absence	1.89±1.17	0.013*	0.67±0.34	0.548	1.97±0.40	0.002*
Presence	2.99±1.19		0.60±0.35		2.44±0.39	

392 *P<0.05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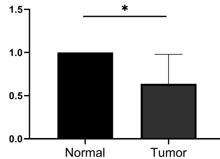
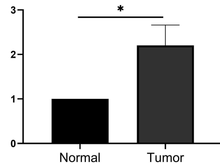
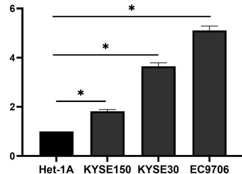
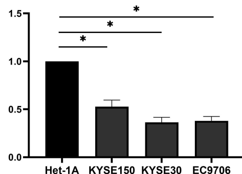
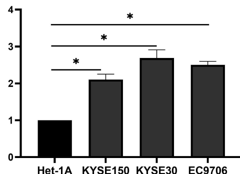
Relative expression of LncRNA NEAT1
*
4
3
2
1
0
Normal
Tumor


B
Relative expression of miR-1299
*
1.5
1.0
0.5
0.0
Normal
Tumor

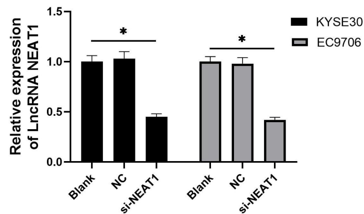

C
Relative expression of MMP2 mRNA
*
3
2
1
0
Normal
Tumor


D
Relative expression of LncRNA NEAT1
*
*
*
6
4
2
0
Het-1A
KYSE150
KYSE30
EC9706


E
Relative expression of miR-1299
*
*
*
1.5
1.0
0.5
0.0
Het-1A
KYSE150
KYSE30
EC9706


F
Relative expression of MMP2 mRNA
*
*
*
4
3
2
1
0
Het-1A
KYSE150
KYSE30
EC9706

A



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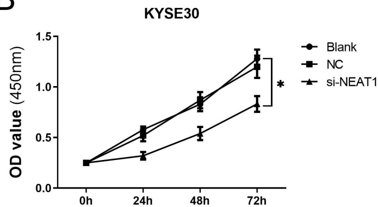

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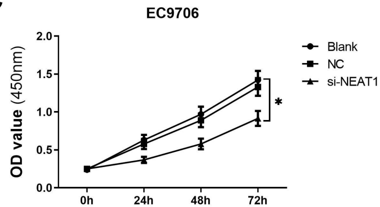

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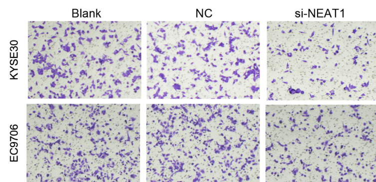

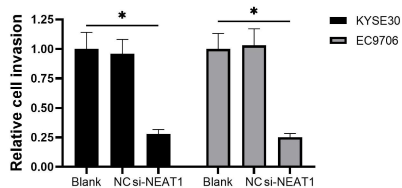

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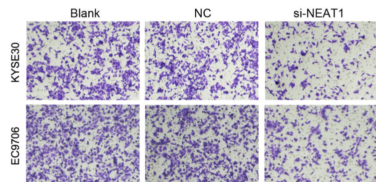
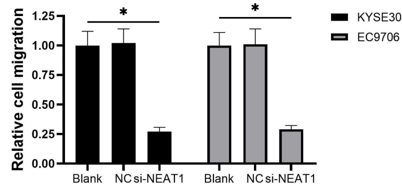
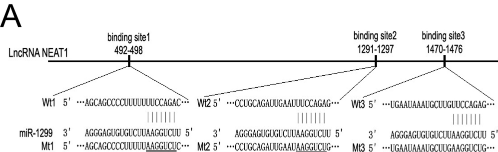
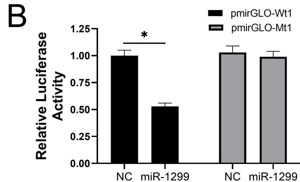
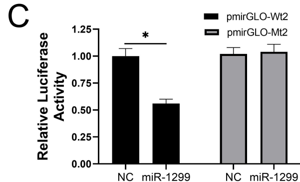
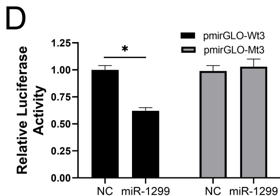





A
LncRNA NEAT1
binding site1
492-498
binding site2
1291-1297
binding site3
1470-1476
Wt1 5′ ···AGCAGCCCCUUUUUUUCCAGAC···
Wt2 5′ ···CCUGCAGAUUGAAUUUCCAGAG···
Wt3 5′ ···UGAAUAAAUGCUUGUUCCAGAG···
miR-1299 3′ AGGGAGUGUGUCUUAAGGUCUU 5′
3′ AGGGAGUGUGUCUUAAGGUCUU 5′
3′ AGGGAGUGUGUCUUAAGGUCUU 5′
Mt1 5′ ···AGCAGCCCCUUUUUAAGGUCUC···
Mt2 5′ ···CCUGCAGAUUGAAUAAGGUCUG···
Mt3 5′ ···UGAAUAAAUGCUUGAAGGUCUG···

B
pmirGLO-Wt1
pmirGLO-Mt1
Relative Luciferase Activity
NC
miR-1299
NC
miR-1299

C
pmirGLO-Wt2
pmirGLO-Mt2
Relative Luciferase Activity
NC
miR-1299
NC
miR-1299

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Relative Luciferase Activity
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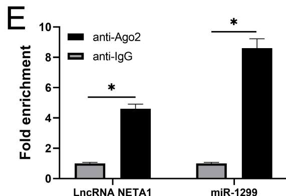
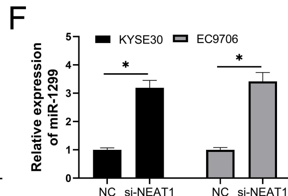
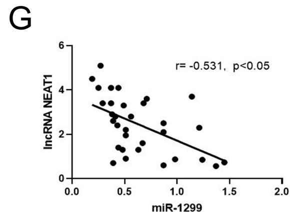
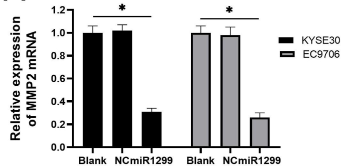
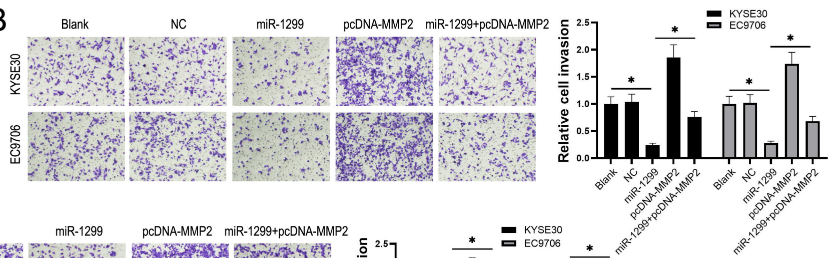
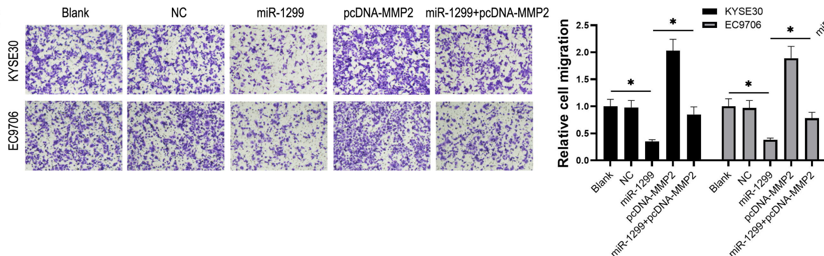
miR-1299
NC
miR-1299

E
anti-Ago2
anti-IgG
Fold enrichment
LncRNA NETA1
miR-1299

F
KYSE30
EC9706
Relative expression of miR-1299
NC
si-NEAT1
NC
si-NEAT1

G
r= -0.531, p<0.05
lncRNA NEAT1
miR-1299

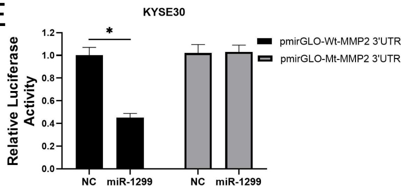


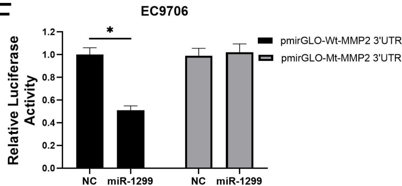




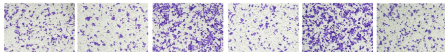
D

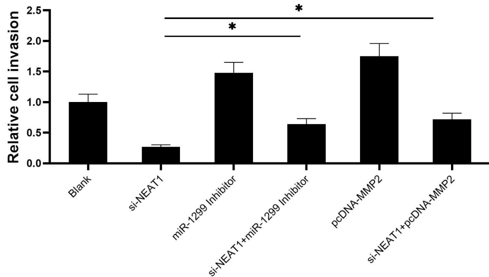
```
Wt MMP2 3'UTR   5'   ...CCUGGUAAUUUAAGAUUCCAGAG...
                                        |||||||
miR-1299        3'      AGGGAGUGUGUCUUAAGGUCUU
                                        |     |
Mt MMP2 3'UTR   5'   ...CCUGGUAAUUUAAGAAAGGUCAG...
```





A





B