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Long Noncoding RNA HOTAIR Promotes Epithelial-Mesenchymal Transition and Is a Suitable Target to Inhibit Peritoneal Dissemination in Human Scirrhous Gastric Cancers

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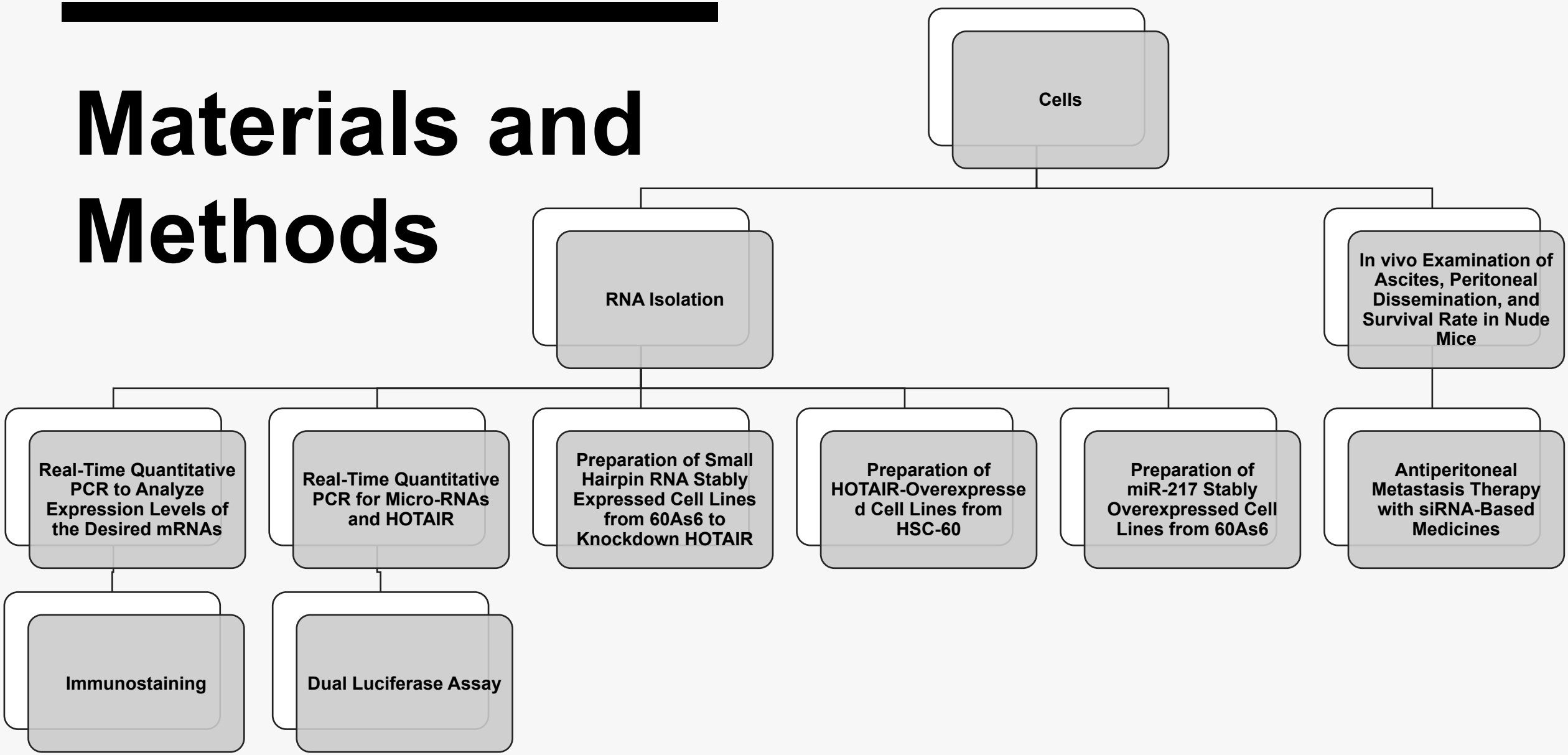
2021

Abstract

- ***Objectives:*** Scirrhous gastric cancer, which accounts for approximately 10% of all gastric cancers, often disseminates to the peritoneum, leading to intractable cases with poor prognosis. There is an urgent need for new treatment approaches for this difficult cancer.
- ***Methods:*** We previously established an original cell line, HSC-60, from a scirrhous gastric cancer patient and isolated a peritoneal-metastatic cell line, 60As6, in nude mice following orthotopic inoculations. In the present study, we focused on the expression of long noncoding ribonucleic acid (RNA) (lncRNA) in the cell lines and investigated the mechanism on peritoneal dissemination.
- ***Results:*** We demonstrated that an lncRNA, HOX transcript antisense RNA (HOTAIR), is expressed significantly more highly in 60As6 than HSC-60 cells. Then, using both HOTAIR knockdown and overexpression experiments, we showed that high-level expression of HOTAIR promotes epithelial-mesenchymal transition (EMT) in 60As6 cells. By luciferase assay, we found that HOTAIR directly targets and binds to miR-217, and that miR-217 directly binds to Zinc finger E-box-binding homeobox 1 (ZEB1). The knockdown of HOTAIR in 60As6 cells significantly reduced the invasion activity and peritoneal dissemination – and significantly prolonged the survival – in the orthotopic tumor mouse model.
- ***Conclusion:*** An EMT-associated pathway (the HOTAIR-miR-217-ZEB1 axis) appears to inhibit peritoneal dissemination and could lead to a novel therapeutic strategy against scirrhous gastric cancer in humans.

Materials and Methods



Results

- HOTAIR Was Expressed at Significantly High Levels in a Metastatic Cell Line, 60As6

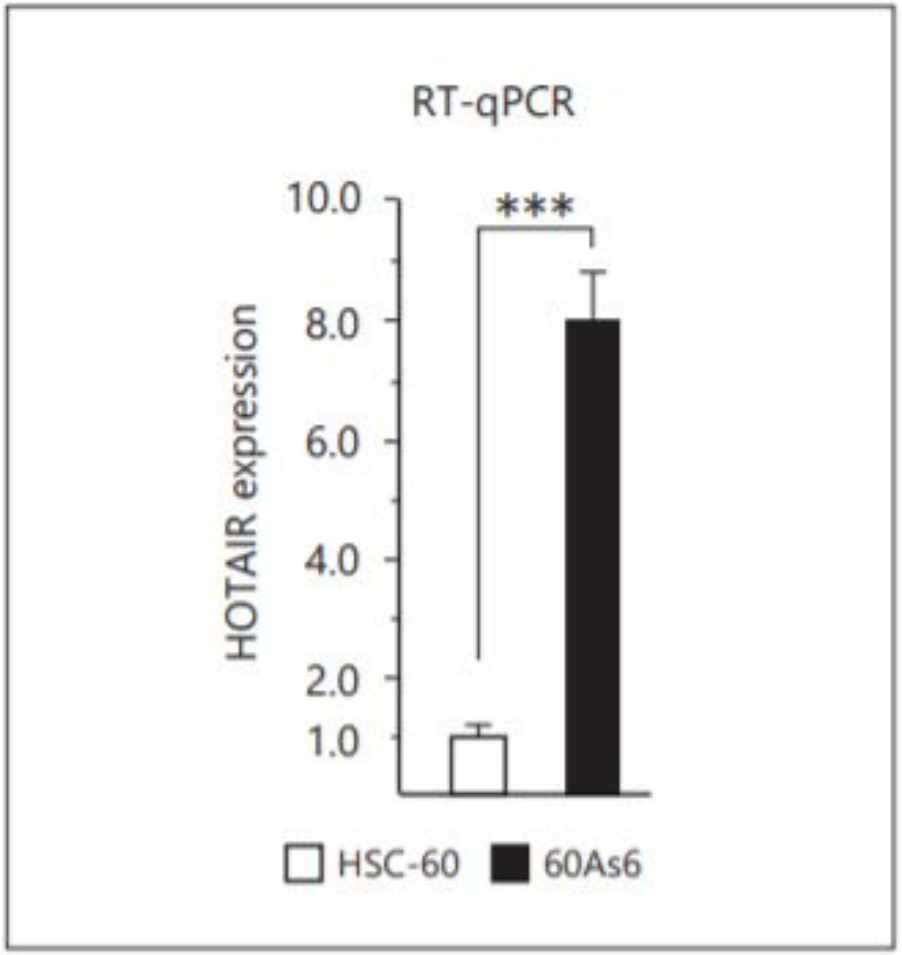


Fig. 1. RT-qPCR analysis of HOTAIR in human scirrhous gastric cancer cell lines HSC-60 and 60As6. White bar, HSC-60; black bar, 60As6. The results are means ± SD ($n = 6$ dishes). ***$p < 0.001$.

Results

- E-Cadherin Was Significantly Decreased, and N-Cadherin and Vimentin Significantly Increased, in 60As6 Cells

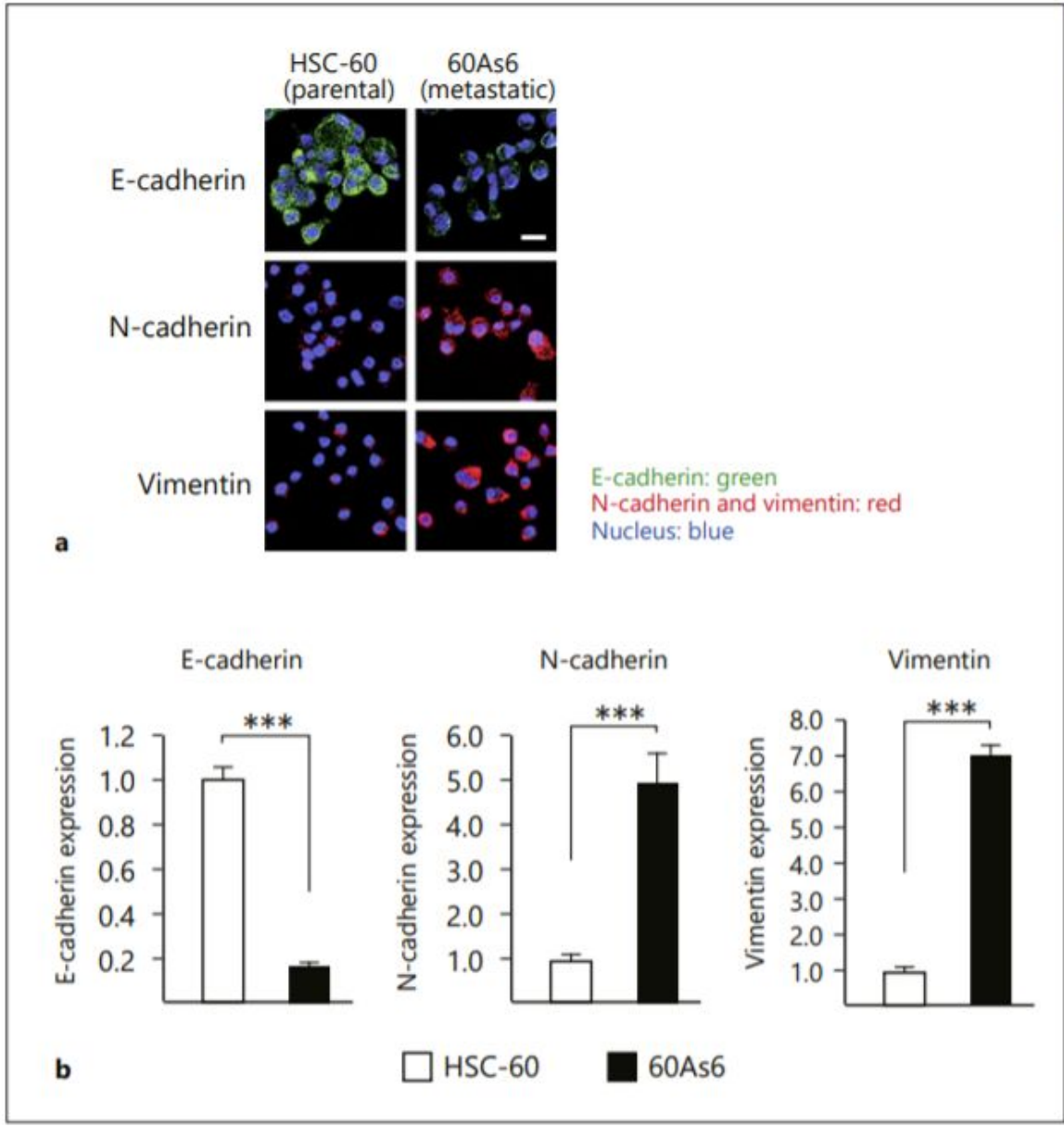


Fig. 2. Immunostaining and RT-qPCR analysis of human scirrhous gastric cancer cell lines HSC-60 and 60As6. **a** Immunostaining of E-cadherin, N-cadherin, and vimentin of the cells. E-cadherin was stained with green fluorescence. N-cadherin and vimentin were stained with red fluorescence. Bar, 50 μm. **b** RT-qPCR analysis of E-cadherin, N-cadherin, and vimentin. White bars, HSC-60; black bars, 60As6. The results are means ± SD ($n = 6$ dishes). ***$p < 0.001$.

Results

- Preparation of a Stable HOTAIR Knockdown Line in 60As6 Cells via shRNA
- The Stable HOTAIR Knockdown Line in 60As6 Cells Reversed EMT Progression

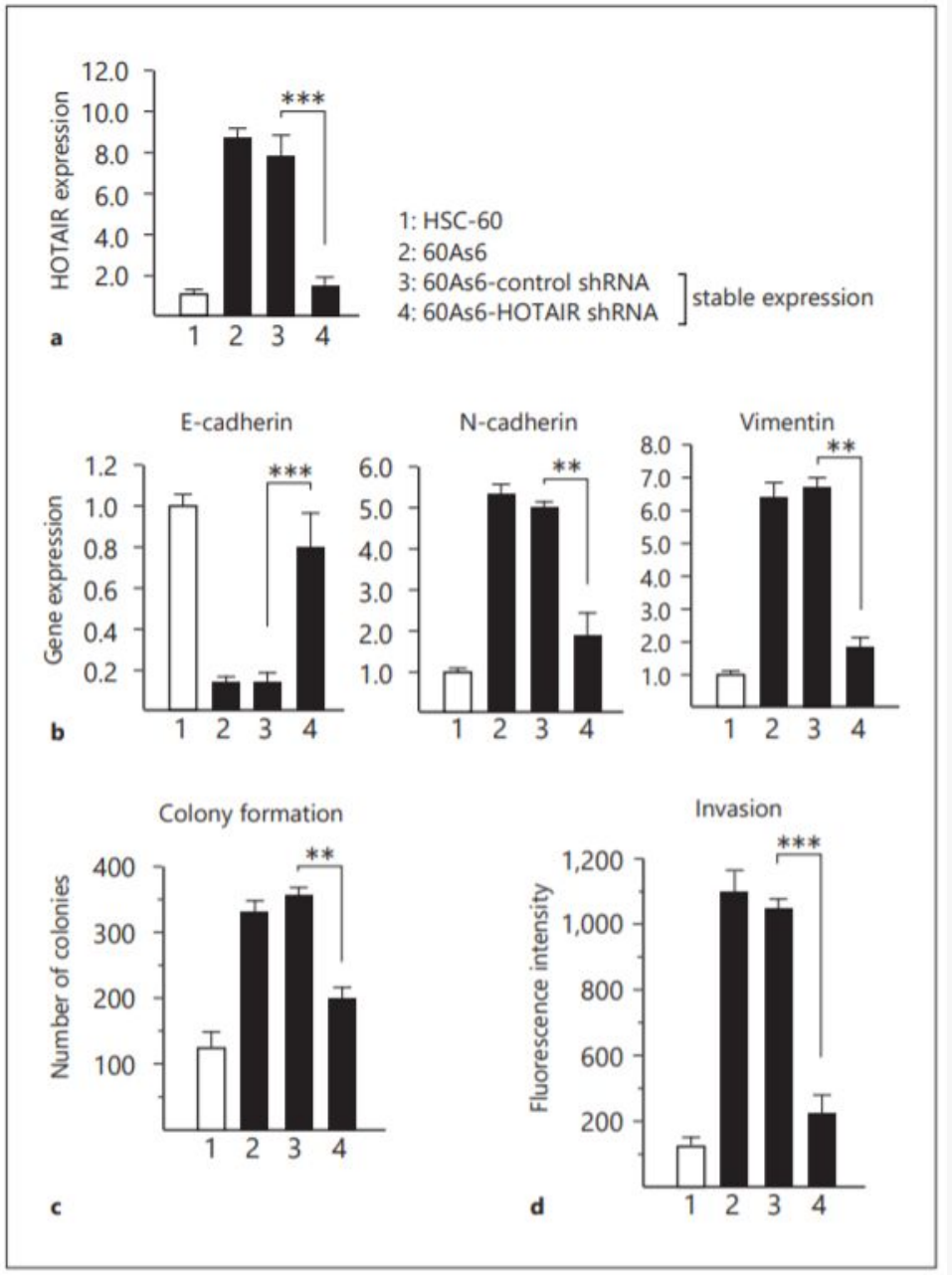


Fig. 3. Effect of HOTAIR knockdown on EMT markers, colony formation, and the invasion property. **a** Preparation of stable HOTAIR knockdown 60As6 cells via specific shRNA vector transfection. As a starting material, 60As6 cells were used and transfected with an shRNA vector to knockdown HOTAIR expression. After the transfection, positive clones were selected using puromycin. RT-qPCR analysis for HOTAIR was performed. The results are means ± SD (n = 6 dishes). ***$p < 0.001$. The names of the 4 kinds of samples are shown in the figure. **b** RT-qPCR analysis of E-cadherin, N-cadherin, and vimentin. The results are means ± SD (n = 6 dishes). *** $p < 0.001$; ** $p < 0.01$. **c** Colony formation assay. The results are means ± SD (n = 6 dishes). **$p < 0.01$. **d** Invasion analysis. The results are means ± SD (n = 6 dishes). *** $p < 0.001$.

Results

- Preparation of a Stable HOTAIR-Overexpressing Cell Line in HSC-60 Cells
- The Stable HOTAIR-Overexpressing Cell Line in HSC-60 Cells Promoted EMT

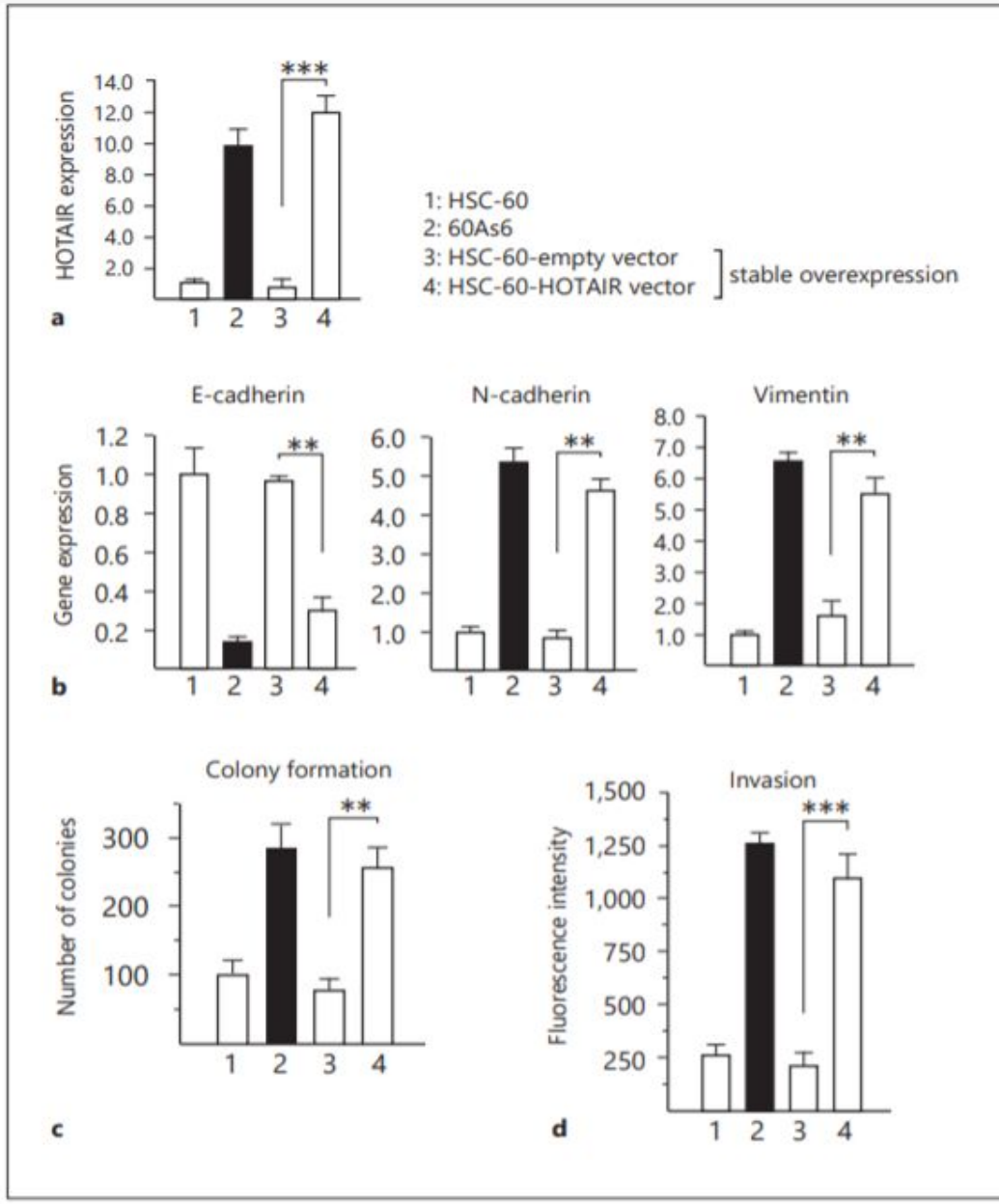


Fig. 4. Overexpression study of HOTAIR in HSC-60. **a** Preparation of stable HOTAIR-overexpressing HSC-60 cells via transfection of a specific expression vector. As a starting material, HSC-60 cells were used. After the transfection, positive clones were selected using geneticin. RT-qPCR analysis for HOTAIR was performed. The results are means ± SD (n = 6 dishes). *** $p < 0.001$. The names of the 4 kinds of samples are shown in the figure. **b** RT-qPCR analysis of E-cadherin, N-cadherin, and vimentin. The results are means ± SD (n = 6 dishes). ** $p < 0.01$. **c** Colony formation assay. The results are means ± SD (n = 6 dishes). ** $p < 0.01$. **d** Invasion analysis. The results are means ± SD (n = 6 dishes). *** $p < 0.001$.

Results

- HOTAIR Targets miR-217 via Direct Binding

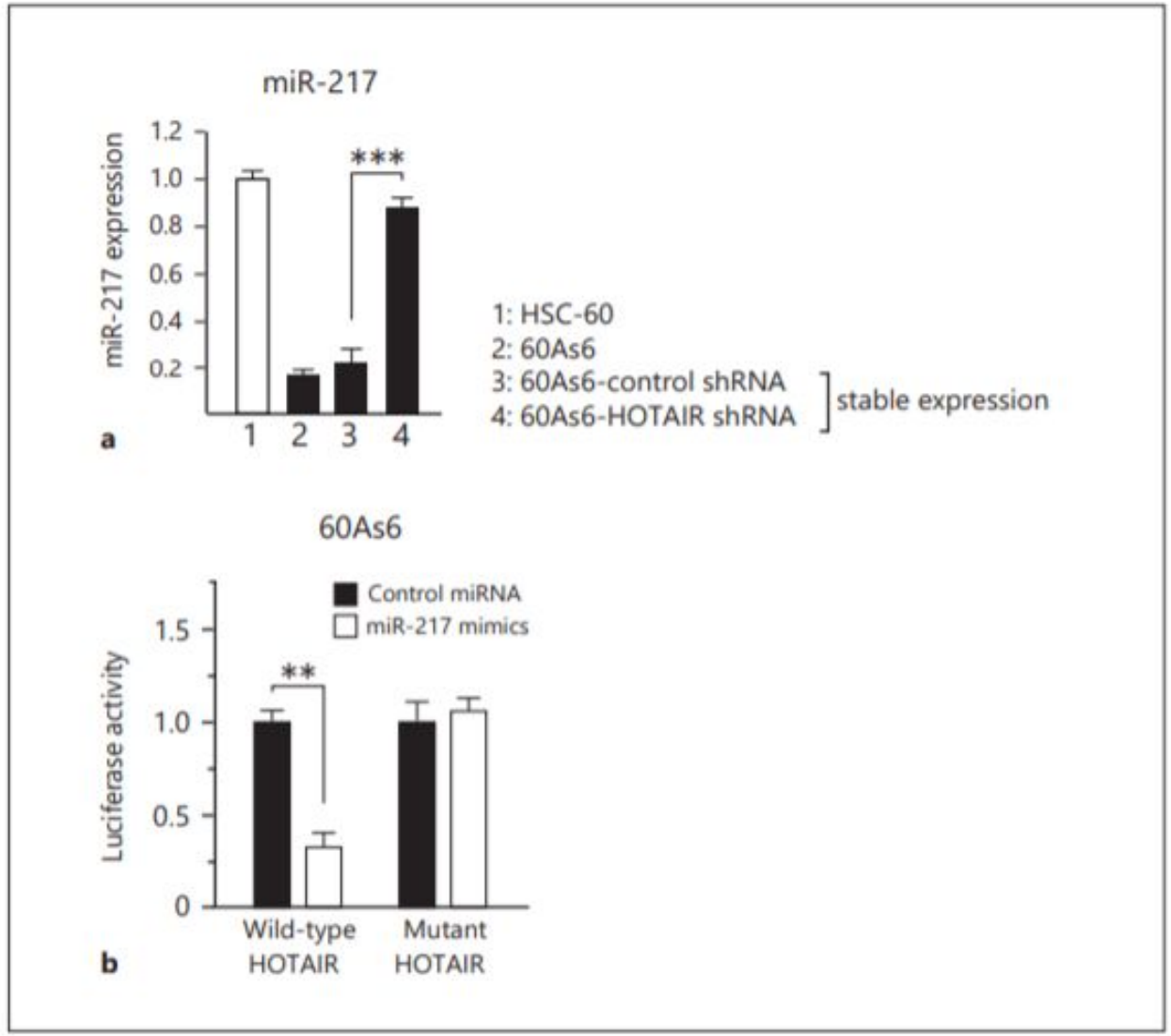


Fig. 5. HOTAIR directly targeted miR-217. **a** The expression level of miR-217 via RT-qPCR analysis. The names of the 4 kinds of samples are shown in the figure. The results are means ± SD ($n = 6$ dishes). *** $p < 0.001$. **b** Luciferase reporter assay. After transient transfection of control miRNA (black bars) or miR-217 mimics (white bars) into 60As6 cells, each cell was transfected with the expression plasmid of wild-type HOTAIR or mutant HOTAIR. Two days later, each cell lysate was evaluated via a dual-luciferase assay. The results are means ± SD ($n = 6$ wells). ** $p < 0.01$.

Results

- miR-217 Directly Targets ZEB1

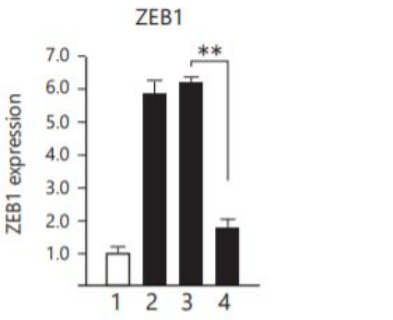


Fig. 6. miR-217 directly targeted ZEB1. **a** Preparation of stably overexpressed 60As6 cells of miR-217 via transfection of a specific expression vector. As a starting material, 60As6 cells were used. After the transfection, positive clones were selected using geneticin. RT-qPCR analysis for miR-217 was performed. The results are means ± SD ($n = 6$ dishes). *** $p < 0.001$. The names of the 4 kinds of samples are shown in the figure. **b** Luciferase reporter assay. The cells (60As6-miR-217) from **a** were transfected with pGL3 vector alone (mock), pGL3-ZEB1-3′-UTR (MUT), or pGL3-ZEB1-3′-UTR as indicated. Two days later, each cell lysate was examined via dual-luciferase assay. The results are means ± SD ($n = 6$ wells). ** $p < 0.01$. **c** RT-qPCR analysis of ZEB1. The results are means ± SD ($n = 6$ dishes). *** $p < 0.001$.

Fig. 7. HOTAIR regulates ZEB1 via miR-217. **a** RT-qPCR analysis of ZEB1. The results are means ± SD ($n = 6$ dishes). ** $p < 0.01$. The names of the 4 kinds of samples are shown in the figure. **b** Schema of our results from HOTAIR to ZEB1 (EMT promotion).

Results

- In vivo Phenotypes

Table 1. Ascites and peritoneal dissemination of nude mice orthotopically inoculated with scirrhous gastric cancer cells

Types of cells inoculated	Ascites volume, mL	Peritoneal dissemination	
		omentum	mesenterium
HSC-60	0.4±0.3	3/15	2/15
60As6	4.6±1.4	15/15	15/15
60As6-control shRNA	4.3±1.1	15/15	14/15
60As6-HOTAIR shRNA	0.9±0.7**	4/15	3/15

In each group, 15 mice were examined. ** $p < 0.01$ versus 60As6-control shRNA.

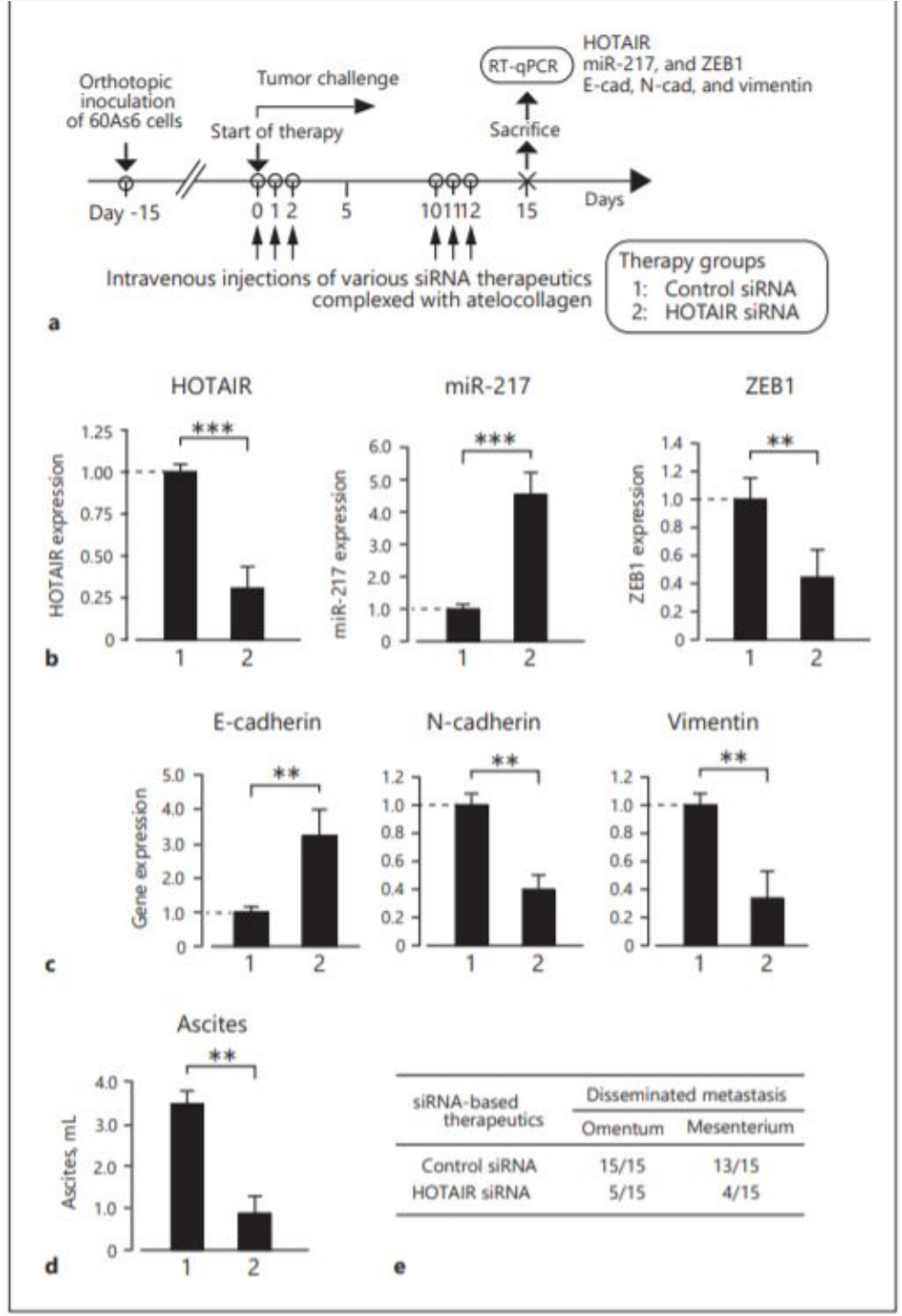
Table 2. Survival days of the nude mice orthotopically inoculated with scirrhous gastric cancer cells

Types of cells inoculated	Survival days of the inoculated mice
HSC-60	104±15
60As6	28±9
60As6-control shRNA	35±11
60As6-HOTAIR shRNA	79±18**

In each group, 15 mice were examined. ** $p < 0.01$ versus 60As6-control shRNA.

Results

- Proposal of Antiperitoneal Dissemination Therapy Targeting HOTAIR



siRNA-based therapeutics	Disseminated metastasis	
	Omentum	Mesenterium
Control siRNA	15/15	13/15
HOTAIR siRNA	5/15	4/15

Fig. 8. Antiperitoneal dissemination therapy with siRNA-based medicines targeting HOTAIR. **a** The 60As6 cells without any vectors, such as shRNA vector or overexpression vector, were orthotopically inoculated. Fifteen days later, various siRNA-based therapeutics (control siRNA or HOTAIR siRNA) complexed with atelocollagen were injected into the tumor-bearing mice (on days 0, 1, and 2 as indicated in the figure). The set of 3 intravenous injections was repeated on each of days 10, 11, and 12. Fifteen mice were used to examine each therapeutic. On day 15, all mice were sacrificed, the primary tumors were excised, and each total RNA was isolated. **b**, **c** RT-qPCR for HOTAIR, miR-217, ZEB1, E-cadherin, N-cadherin, and vimentin. The results are means ± SD (n = 15 mice). *** $p < 0.001$; ** $p < 0.01$. Lane 1, control siRNA; lane 2, HOTAIR siRNA. **d** Ascites volume in the mice. The results are means ± SD (n = 15 mice). ** $p < 0.01$. Lane 1, control siRNA; lane 2, HOTAIR siRNA. **e** Examination of peritoneal dissemination in the mice.

Results

- HOTAIR-miR-217-ZEB1 Expression Levels in Cancer Tissues from Patients

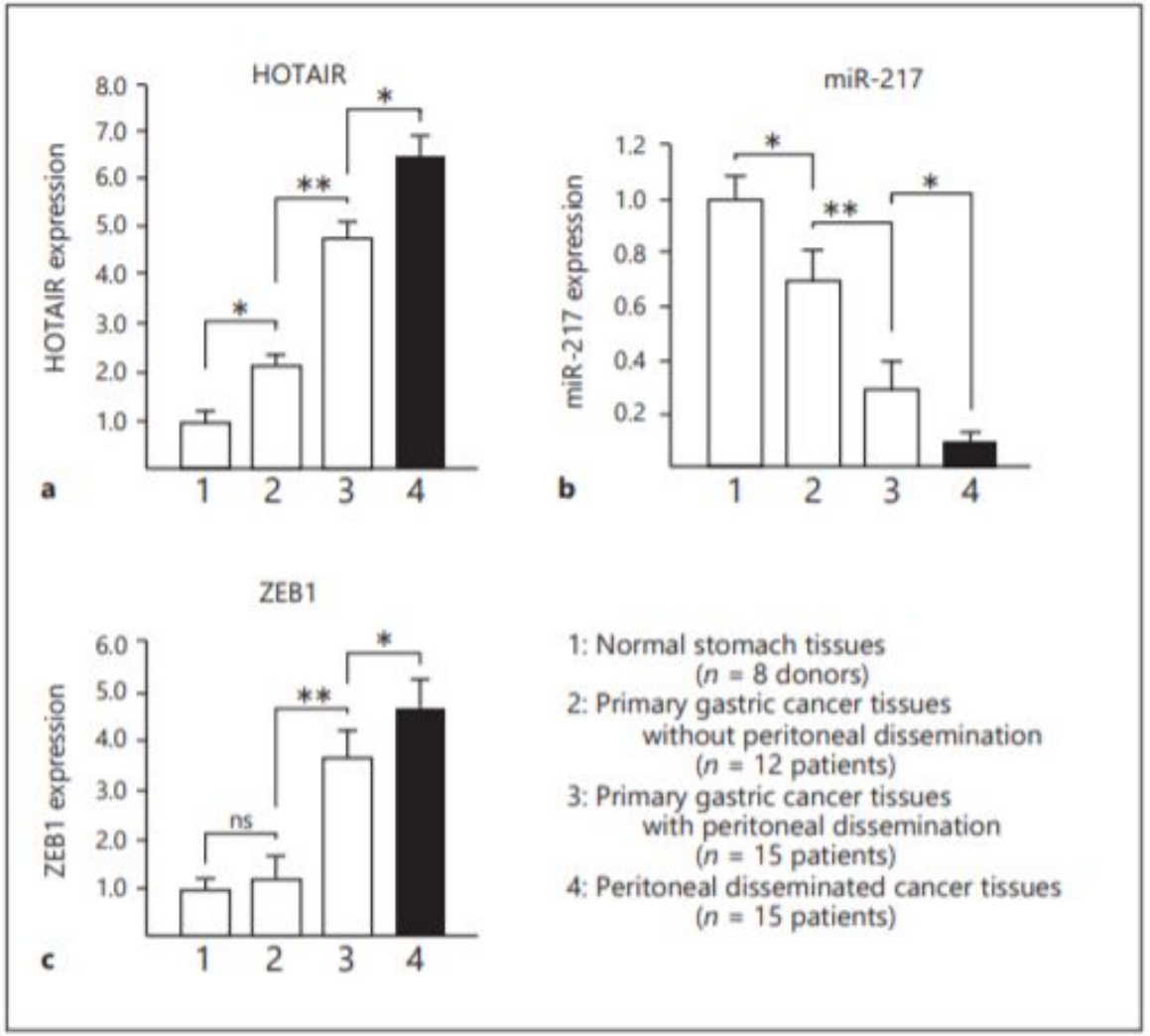


Fig. 9. Expression levels of HOTAIR (**a**), miR-217 (**b**), and ZEB1 (**c**) in clinical samples from gastric cancer patients. Each sample category (from 1 to 4) is shown in the figure. RT-qPCR of each clinical sample was performed. The numbers of samples are also shown. $^{**}p < 0.01$; $^{*}p < 0.05$. ns, not significant.

Discussion

- Thus, in 60As6 cells, our highly metastatic line derived from a patient, the expression of HOTAIR was significantly increased (Fig. 1) compared with that in the parental line with low metastatic properties (HSC-60).
- We also demonstrated using the patient clinical samples that the HOTAIR expression was significantly higher in primary cancer tissues from patients with peritoneal metastasis than in the cancer tissues from patients without peritoneal metastasis (Fig. 9). The HOTAIR expression was further increased in the peritoneal cancer tissues (Fig. 9). These findings are important in 2 ways.

(i) They suggest that HOTAIR expression could feasibly be used to clinically diagnose peritoneal metastasis of scirrhous gastric cancers.

(ii) They suggest that HOTAIR can be targeted to inhibit peritoneal metastasis of scirrhous gastric cancers (opening the possibility of a novel antiperitoneal dissemination therapy).

- Elevated HOTAIR in 60As6 contributes to the promotion of EMT (Fig. 2-4), and the signals of elevated HOTAIR are transduced to downregulate the miR-217 level and further to dysregulate ZEB1 expression (as a result, upregulation of ZEB1; Fig. 5-7).

Discussion

- That upregulation of ZEB1 promotes EMT and cancer metastasis is well known. In our in vivo study, we obtained 2 excellent results as follows.
  (i) When the 60As6-HOTAIR shRNA line (stably expressed line) was orthotopically inoculated into the stomach wall in nude mice, we observed an inhibition of peritoneal dissemination and prolonged survival rate (Tables 1, 2).
  (ii) In tumor-bearing mice with orthotopic inoculation of 60As6 without any transfection of the shRNA, when we intravenously injected the complex of siRNA targeting HOTAIR and atelocollagen, a marked antiperitoneal dissemination effect was shown (Fig. 8).
- In the treated mice, the HOTAIR expression was reduced to increase miR-217, and to decrease ZEB1 (Fig. 8), as observed in the in vitro study. Further, HOTAIR knockdown in the mice led to a reversal of the EMT status (Fig. 8). We also showed that the overall survival rate of the treated mice was significantly longer (73 ± 13 days) than that of the control mice (36 ± 9 days), as shown in Table 3.

Table 1. Ascites and peritoneal dissemination of nude mice orthotopically inoculated with scirrhous gastric cancer cells

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Table 2. Survival days of the nude mice orthotopically inoculated with scirrhous gastric cancer cells

Types of cells inoculated	Survival days of the inoculated mice
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60As6-control shRNA	35±11
60As6-HOTAIR shRNA	79±18**

In each group, 15 mice were examined. ** $p < 0.01$ versus 60As6-control shRNA.

Table 3. Significantly prolonged survival days of antiperitoneal dissemination therapy with siRNA-based medicines against HOTAIR

siRNA-based medicines	Survival days of the treated mice
Control siRNA	36±9
HOTAIR siRNA	73±13**

Each therapy group included 15 mice. ** $p < 0.01$ versus control siRNA.

Conclusions

- We demonstrated that high expression of HOTAIR in 60As6 promotes EMT to assist the peritoneal dissemination of scirrhous gastric cancers. The EMT-associated pathway (HOTAIR-miR-217-ZEB1 axis) might be targeted to inhibit peritoneal dissemination as a novel strategy for the treatment of scirrhous gastric cancers (Fig. 10).

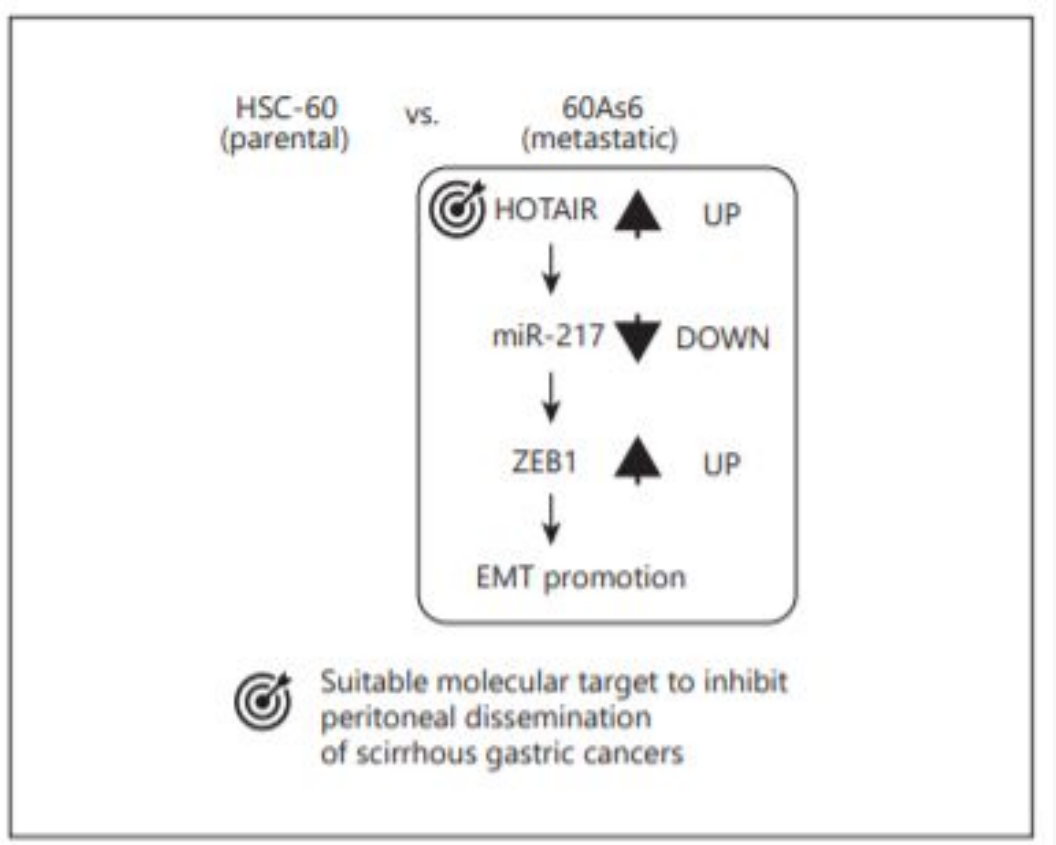

Fig. 10. HOTAIR is a suitable target for the inhibition of peritoneal dissemination in human scirrhous gastric cancers.

Questions for discuss

1. What is the potential clinical significance of the presented results?
2. What are the prospects for this study?
3. What is the clinical relevance of HOTAIR for the diagnosis of scirrhoid gastric cancer?