Introduction

As the first oncogene identified from human tumours, Ras is one of the most well-known oncogenes aberrantly activated in tumourigens is [1-3]. Ras is a cytoplasmic membrane-associated small GTPase which, in response to various stimuli, regulates multiple signalling pathways important for cell proliferation and survival. It functions through the oscillation between the GTP-bound active form and the GDP-bound inactive form. After GTP binding, Ras undergoes a conformational change which enables it to activate downstream effectors, such as Raf. Ras activity is tightly regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Upon the binding of growth factors to cognate receptors, Ras-GEFs, like SOS, can form a complex with Ras and induce the dissociation of Ras with GDP to allow GTP binding. In contrast, GAPs trigger the hydrolysis of bound GTP into GDP and attenuate Ras activity.

The Ras signalling pathway is believed to be aberrantly activated in most if not all tumours. Aberrant activation of Ras signalling is often achieved through genetic changes, mainly mutations that render Ras constitutively active in the GTP-bound form. Such oncogenic mutations have been frequently found in human cancers. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%) and the thyroid (50%)[3]. However, Ras mutations are rarely found in gastric cancer, suggesting that other mechanisms may be responsible for aberrant Ras activation in this type of cancer.

It was recently found that membrane-actin
linker protein ezrin is important for Ras activation [4-5]. Ezrin can interact with membrane-associated adhesion molecules through its N-terminus and the actin cytoskeleton through its C-terminus. By doing so, ezrin plays a critical role in the remodelling of the cortical actin cytoskeleton, which is important for the assembly of the ‘signalosome’ complex that promotes the activation of Ras by SOS [4-5]. Ras cannot be activated in the absence of ezrin. Moreover, ezrin mutants that are unable to interact with F-actin can lead to the disruption of SOS/Ras complex formation, with the consequent inhibition of Ras activation [6]. The tumour suppressor merlin functions in a similar way as these mutants of ezrin, since it is highly homologous to ezrin in the N-terminus, but lacks the actin-binding domain conserved in ezrin [4, 7-8]. The interplay between merlin and ezrin is therefore important to regulate Ras activation. Thus, ezrin upregulation, similar to the loss of merlin function, may represent another mechanism of aberrant Ras activation in human cancers. Ezrin is indeed highly expressed in multiple cancers and ezrin overexpression in tumours is associated with poor survival of cancer patients [9-11]. However, whether ezrin overexpression plays a role in the development of gastric cancer and how it is upregulated in the progression of gastric cancer remain unknown.

**Materials and methods**

**Cell lines and tissue samples**

Unless specifically indicated, cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (FBS) and incubated at 5% CO₂, 37°C and 95% humidity. All primary tissues were obtained from the Endoscopy Centre of the Prince of Wales Hospital, The Chinese University of Hong Kong. All specimens were immediately snap-frozen in liquid nitrogen and stored at –80°C until further processing.

**SiRNA and miRNA precursor transfection**

Ezrin depletion and microRNA re-expression were achieved by transfection with siRNAs and miRNA precursors (Qiagen, Hilden, Germany), respectively. Cells were seeded in 12-well plates (1×10⁵/well) and transfected with siRNA duplexes or miRNA mimic (10 nM) using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instructions. Cells were harvested for RNA and protein extraction after 72 hours.

**Cell growth assay**

Cell growth was measured by a non-radioactive proliferation assay based on the ability of metabolically active cells to convert 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) into formazan using the CellTiter 96® AQüeous Assay kit (Promega, Madison, WI, USA). Briefly, cells were seeded in 12-well plates and transfected with ezrin siRNA or miR-204 mimic for 48 hours. Cells were then harvested and re-seeded into 96-well plates. After 24 hours, the quantity of formazan was measured at 490 nm absorbance after one hour of incubation with CellTiter 96® AQüeous One Solution Reagent following the instructions provided.

**Cell migration assay**

The cell migration assay was performed using the QCM™ 24-well colorimetric cell migration assay (Millipore, Billerica, MA, USA). Transfected AGS cells (a human gastric cancer cell line) were serum starved for 24 hours and detached with trypsin/EDTA. Cells (2.5×10⁴) in serum-free medium were introduced to the upper chamber of the migration insert with 10% FBS-containing medium in the lower chamber and incubated for 24 hours at 37°C. Cells which had migrated to the bottom of the insert membrane were observed under a microscope and dissociated from the membrane for colourimetric measurement at a wavelength of 560 nm in accordance with the manufacturer’s instructions.

**Total RNA extraction**

Cell pellets or tissues were homogenised in Trizol reagent (Invitrogen). Total RNA containing small RNA was extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. DNase treatment was carried out to remove any contaminating DNA. The concentrations of all RNA samples were quantified by NanoDrop 1000 (Nanodrop, Wilmington, DE, USA).

**mRNA detection**

The reverse transcription reaction was performed using 1 μg of total RNA with the High
MicroRNA-204 downregulation in gastric cancer

Table 1. Sequence of primers used

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ezrin-F</td>
<td>TGTGGTACTTTGGCCTCCAC</td>
</tr>
<tr>
<td>Ezrin-R</td>
<td>GTTCTGTTGATGTCCTGG</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>GGAGTCAACGGGATTGTG</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>GTGATGGGATTTCATTG</td>
</tr>
<tr>
<td>miR-204</td>
<td>CCTTCCCTTGTACCTCAT</td>
</tr>
<tr>
<td>U6</td>
<td>ACGCAAATTCGAAAGCGTT</td>
</tr>
<tr>
<td>EZR-WT-sense</td>
<td>AATGCACTAGTTGATCCTGATAATACAGATTGTAAACATTAGTTGATTCAGGATCCAC-TAGTGCATT</td>
</tr>
<tr>
<td>EZR-WT-antisense</td>
<td>GCATTAAGCTCTTTCTCTCTTTTTTTAAACTAATGTTACATCTGATTATCGGATCCAC-TAGTGCATT</td>
</tr>
<tr>
<td>EZR-MT-sense</td>
<td>AATGCACTAGTTGATCCTGATAATACAGATTGTAAACATTAGTTGATTCAGGATCCAC-TAGTGCATT</td>
</tr>
<tr>
<td>EZR-MT-antisense</td>
<td>GCATTAAGCTCTTGATCCTGATAATACAGATTGTAAACATTAGTTGATTCAGGATCCAC-TAGTGCATT</td>
</tr>
<tr>
<td>EZR-Deletion-sense</td>
<td>AATGCACTAGTTGATCCTGATAATACAGATTGTAAACATTAGTTGATTCAGGATCCAC-TAGTGCATT</td>
</tr>
<tr>
<td>EZR-Deletion-antisense</td>
<td>GCATTAAGCTCTTCAAATCTGATTATCGGATCCAC-TAGTGCATT</td>
</tr>
</tbody>
</table>

Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the SYBR Green Master Mix Kit and ABI PRISM 7900 Real-Time PCR System (Applied Biosystems). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control of RNA integrity. The primers used for ezrin and GAPDH RT-PCR are listed in Table 1.

MicroRNA quantification by real-time RT-PCR

SYBR green qRT-PCR assay was used for miRNA quantification. In brief, total RNA was polyadenylated and reverse transcribed to cDNA using the miScript Reverse Transcription kit (Qiagen). Real-time qPCR was performed using the miScript SYBR Green PCR kit (Qiagen) in ABI PRISM 7900 Real-Time PCR System (Applied Biosystems). The expression levels of the miRNAs were normalized to U6. The miRNA-specific primer sequences are listed in Table 1.

The amplification was performed at 95°C for 15 minutes, followed by 40 cycles of 94°C for 15 seconds, 55°C for 30 seconds and 70°C for 30 seconds. At the end of the PCR cycles, melting curve analyses as well as agarose electrophoresis were performed to validate the specific generation of the expected PCR product.

Immunohistochemical staining with tissue microarray

Tissue microarrays containing 150 gastric carcinoma tissues were constructed and ezrin expression was detected with immunohistochemical staining. Briefly, following antigen retrieval, the sections were blocked with normal goat serum (Dako, Glostrup, Denmark) and then incubated with ezrin-specific primary antibody (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The detection of the antigen-antibody complex was performed using a goat anti-rabbit secondary antibody and the Streptavidin-HRP Systems kit (Dako).

Scoring of ezrin staining

Immunohistochemical staining of ezrin was evaluated based on the proportion of ezrin-positive cells and average staining intensity for the whole tissue section. The percentage of positive cells was scored on a scale of 1 to 3: 1, less than 25%; 2, 25% to 50% and 3, more than 50%. Staining intensity was scored on a scale of 1 to 3: 1, negative or weak staining; 2, moderate staining and 3, strong staining. The two scores were then multiplied, resulting in an immunoreactivity score (IRS) value ranging from 1 to 9. Ezrin expression status were then grouped into three categories based on IRS values: low, IRS 1–3; moderate, IRS 4–6 and high, IRS 7–9.

Construction of microRNA reporter vector

The 3' UTR of ezrin mRNA containing intact miR-204 binding sequences was inserted immediately downstream of a CMV promoter-driven firefly luciferase cassette in a pMIR-reporter vector according to the protocol provided (Ambion, Austin, TX, USA). Restriction enzymes
SpeI and HindIII were used. To make control vectors, two mutant constructs were generated by either point mutations or deletion of the seed sequence of miR-204-ezrin interaction. The sequences of the oligonucleotides used are listed in Table 1.

**Luciferase activity assay**

Cells (1×10⁵) were co-transfected with pMIR-Luciferase-ezrin-3′-UTR and pRL-CMV-Renilla (Promega) constructs in the presence of 30 pmol of miR-204 mimic or control oligonucleotides. Forty-eight hours after transfection, the activities of firefly luciferase and renilla luciferase were measured using the Dual-Glo™ luciferase assay system (Promega) as described by the manufacturer. Relative luciferase activity was normalized with renilla luciferase activity and then compared with the pMIR-reporter vector control.

**Ras activity assay**

The Ras activity assay was carried out using the Ras Activation Assay Kit (Millipore). Active Ras was pulled down with purified GST-Raf-RBD agarose beads by incubating cell lysates with GST-Raf-RBD pre-bound to glutathione-sepharose. Bound proteins were subjected to SDS-PAGE and immunoblotting analysis with an anti-Ras antibody.

**Immunoblotting**

Twenty-five microgram of protein was resolved on 10–12.5% SDS-PAGE minigels and transferred onto Hybond C nitrocellulose membranes (Amersham Life Science, Buckinghamshire, UK). Blocked membranes were probed with primary antibodies in blocking buffer overnight at 4°C. Finally, membranes were incubated with secondary antibodies conjugated with HRP (horseradish peroxidase) and signals were visualised with enhanced chemiluminescence (Amersham Life Science). Membranes were re-probed with antibodies against b-actin (Cell Signaling Technology) or GAPDH (Abcam, Cambridge, MA, USA) as the loading control.

**Statistical analysis**

The difference in the expression of miR-204 between tumour and adjacent non-tumour tissues was analyzed by the Wilcoxon matched pairs test. The χ² tests were used to analyze the association of patient characteristics with ezrin expression. The probability of overall survival was calculated with the Kaplan-Meier method and differences between curves were evaluated with the log-rank test. Relative risks of death associated with ezrin expression and other predictor variables were estimated by the univariate Cox proportional hazards model. Multivariate Cox models were also constructed to estimate the relative risk for ezrin expression, with adjustments of age, gender, Lauren type, differentiation and TNM stage. All analyses were performed using SPSS for Windows, version 14.0. A p value < 0.05 was taken as statistically significant.

**Results**

**Ezrin is upregulated in gastric cancer**

In order to ascertain whether ezrin is relevant to gastric carcinogenesis, we began by determining the expression of ezrin in a panel of human gastric carcinoma cell lines. Ezrin was found to be highly expressed in all gastric carcinoma cell lines (Figure 1A). We then examined ezrin expression in primary gastric carcinoma tissues with immunohistochemical staining. Ezrin expression was much higher in carcinoma tissues than in adjacent non-tumour tissues (Data not shown), indicating that ezrin may be involved in gastric cancer development.

**Ezrin upregulation is important to Ras activation in gastric cancer cells**

Next, we investigated the biological relevance of ezrin upregulation in gastric cancer cells. Down-regulation of ezrin expression using ezrin-specific siRNAs reduced Ras activity as well as Erk1/2 phosphorylation (Figure 1B). Consistent with this, cell growth and cell migration were also significantly inhibited after ezrin depletion (Figures 1C and 1D).

**Ezrin upregulation is clinically relevant to gastric carcinogenesis**

To further illustrate the clinical relevance of ezrin expression to gastric carcinogenesis, we examined ezrin expression by tissue microarray analysis. Among 150 gastric cancer cases, 33 (22.0%) cases showed low ezrin expression, 92 (61.3%) cases showed moderate ezrin expres-
MicroRNA-204 downregulation in gastric cancer

sion and 25 (16.7%) cases showed high ezrin expression. The association of ezrin expression with various clinicopathologic features is summarized in Table 2. Ezrin expression was significantly correlated with Lauren type \( (p<0.01) \) and differentiation status \( (p<0.05) \). In addition, both univariate and multivariate Cox regression analysis revealed a significant association of ezrin expression with the survival of gastric cancer patients (Table 3). Higher ezrin expression indicated a poorer outcome in both overall (all stages) (Figure 2A) and early (stage I and II) gas-

Table 2. Clinicopathologic features of ezrin expression in gastric cancer

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Ezrin expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
</tr>
<tr>
<td>Age</td>
<td>Mean±SD</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>24 (26.09)</td>
</tr>
<tr>
<td>F</td>
<td>9 (16.36)</td>
</tr>
<tr>
<td>H. pylori infection</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>18 (21.43)</td>
</tr>
<tr>
<td>Negative</td>
<td>13 (27.08)</td>
</tr>
<tr>
<td>Lauren type</td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>7 (12.73)</td>
</tr>
<tr>
<td>Intestinal</td>
<td>26 (30.23)</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>4 (57.14)</td>
</tr>
<tr>
<td>Moderate</td>
<td>17 (28.33)</td>
</tr>
<tr>
<td>Poor</td>
<td>9 (12.50)</td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6 (31.58)</td>
</tr>
<tr>
<td>II</td>
<td>7 (33.33)</td>
</tr>
<tr>
<td>III</td>
<td>10 (17.54)</td>
</tr>
<tr>
<td>IV</td>
<td>9 (17.30)</td>
</tr>
</tbody>
</table>

Figure 1. Effect of ezrin knock-down on Ras activation, cell growth and cell migration. A. Ezrin expression in a panel of gastric cancer cell lines was determined by immunoblotting. Normal (non-tumour, non-inflammation) stomach tissue was used as the reference. B. Ras activity and Erk1/2 phosphorylation in gastric cancer cells (NCI-N87) before and after ezrin knockdown were determined by immunoblotting. Active Ras indicates Ras-GTP pulled down by Raf-RBD. MTS assay (C. NCI-N87 and AGS cells) and cell migration assay (D. AGS cells) were used to determine the effect of ezrin knock-down on cell growth and cell migration, respectively. A representative experiment in triplicate is shown as mean±SD. The asterisks indicate a statistically significant difference (Student’s t-test, \( p<0.05 \)).
MicroRNA-204 downregulation in gastric cancer

MiR-204 is downregulated in gastric cancer

Ezrin was found to be upregulated at the protein level in gastric cancer (Figure 1A and data not shown). However, there was no significant difference in the ezrin mRNA level in primary gastric carcinomas and adjacent non-tumour tissues (data not shown), suggesting a post-transcriptional mechanism of ezrin upregulation in gastric carcinogenesis. In this study, we wanted to determine whether ezrin upregulation in gastric cancer is caused by microRNA deregulation. Bioinformatics analysis revealed several microRNAs may target ezrin expression, such as miR-204, miR-211 and miR-183. Only miR-204 was significantly downregulated in both gastric cancer cell lines (Figure 3A) and primary gastric carcinoma tissues (Figure 3B). In addition, the

<table>
<thead>
<tr>
<th>Ezrin expression</th>
<th>n</th>
<th>MST (months)</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RR (95% CI)</td>
<td>p</td>
</tr>
<tr>
<td>Low</td>
<td>33</td>
<td>67.7 (51.5–84.0)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>92</td>
<td>42.2 (32.4–52.0)</td>
<td>2.01 (1.18–3.44)</td>
<td>0.01 1.86 (1.06–3.26)</td>
</tr>
<tr>
<td>High</td>
<td>25</td>
<td>19.3 (10.6–27.9)</td>
<td>3.46 (1.84–6.52)</td>
<td>&lt;0.001 2.37 (1.24–4.56)</td>
</tr>
</tbody>
</table>

CI, confidence interval; RR, relative risk; MST, mean survival time.
MicroRNA-204 downregulation in gastric cancer

complementary site in ezrin 3'-UTR for miR-204 base pairing was highly conserved in different species (Figure 4A). Thus, we focused on miR-204 in this study.

MiR-204 directly targets ezrin expression

Introduction of miR-204 mimic into gastric cancer cells with miR-204 downregulation reduced ezrin expression (Figure 4B). To further support the direct interaction of miR-204 with 3'-UTR of ezrin, we constructed a luciferase reporter system in which luciferase expression was controlled by wild-type, mutated ezrin 3'-UTR or ezrin 3'-UTR without miR-204-interacting sequence (deletion) (Figure 4C). In mutated ezrin 3'-UTR, several nucleotides in the seed region were mutated to disrupt the interaction with miR-204. Indeed, miR-204 could suppress the expression of luciferase regulated by wild-type but not mutated ezrin 3'-UTR or ezrin 3'-UTR deficient with miR-204-binding sequence (Figure 4D), demonstrating a direct effect of miR-204 on ezrin 3'-UTR.

MiR-204 downregulation is important to the activation of Ras signalling

To further confirm the effect of miR-204 on ezrin expression, we set out to study the effect of miR-204 on Ras activation and cellular behaviors, such as cell growth and cell migration. Similar to the effect of ezrin depletion induced by ezrin-specific siRNA, miR-204 inhibited Ras activation (Figure 5A), and suppressed cell growth (Figure 5B) and cell migration (Figure 5C).

Discussion

Aberrant Ras activation is believed to play a critical role in many if not all cancers. Point mutations that render Ras constitutively active have been frequently found in many tumours, such as pancreatic carcinoma. However, such oncogenic Ras mutations have rarely been detected in gastric cancer. Many genetic and epigenetic changes as well as environmental factors may contribute to aberrant Ras activation in gastric cancer instead of oncogenic Ras mutations. For example, growth factor receptors, such as epithelial growth factor receptors (EGFRs), are overexpressed via gene amplification in gastric cancer [12]. In addition, Helicobacter pylori infection, one of the risk factors for gastric cancer, can activate Ras through EGFR transactivation [13]. Herein, we have presented another mechanism for aberrant Ras activation in gastric carcinogenesis.

As the founder member of the ezrin-radixin-moesin family, ezrin functions as a link between the plasma membrane and the cortical actin cytoskeleton [14-15]. The interaction of ezrin with the actin cytoskeleton is instrumental to many cellular processes, including cell signalling.

Figure 4. Direct targeting of ezrin expression by miR-204. Predicted interaction of miR-204 (grey) with ezrin 3'-UTR (black) is shown in A. B. Ezrin expression in NCI-N87 cells before and after miR-204 re-expression was determined by immunoblotting. The schematic of microRNA luciferase reporter construct is shown in C. WT: wild type; MT: mutated type. The seed sequence is underlined. D. Expression of luciferase in the presence of miR-204 or control oligonucleotides was determined by the luciferase reporter assay. A representative experiment is shown as mean±SD. The asterisk indicates a statistically significant difference (Student’s t-test, p<0.05).
MicroRNA-204 downregulation in gastric cancer

Ezrin upregulation is associated with poor outcome in some cancers, such as colorectal cancer and soft tissue sarcoma [10-11]. In this study, we found that strong ezrin expression was associated with poor survival of gastric cancer patients (Figure 2A). Interestingly, ezrin expression had some value in the prognosis prediction of early (stage I and II) gastric cancer (Figure 2B), suggesting that more intensive treatment should be given to patients with strong ezrin expression, even at the early stage of the disease.

Many genetic and epigenetic alterations can contribute to aberrant Ras activation during the stepwise process of cancer development [2, 4, 7, 18-20]. Recently, miRNAs, known as non-coding small RNAs, have emerged as important regulators of development and disease progression by controlling processes such as cell fate determination [21-26]. The founding member of the miRNA family, let-7 (lethal-7), is required for the timing of cell fate determination in Caenorhabditis elegans [27-28]. Ras has been identified as the direct target regulated by let-7 [29]. Herein, we have presented another miRNA that regulates Ras activity. Ezrin, the upstream regulator of Ras activation, contains a miR-204 complementary site in its 3’-UTR, subjecting it to miRNA-mediated gene suppression (Figure 4). Ezrin was found to be upregulated in gastric cancer cells. In contrast, miR-204 was downregulated in gastric cancer cells and miR-204 mimic inhibited ezrin expression, Ras activation and cell growth (Figure 5). All of these data strongly demonstrate that ezrin is a genuine direct target of miR-204.

In conclusion, we found that ezrin overexpression is important to Ras activation and associ-
ated with poor outcome in gastric cancer. By directly targeting ezrin expression, miR-204 functions as a new miRNA regulator of Ras activation, and its downregulation represents a novel mechanism for aberrant Ras activation in cancer development.

Acknowledgement

The project was supported by Program for New Century Excellent Talents in University, National Natural Science Foundation of China (81071652) and Fundamental Research Funds for the Central Universities to HJ, and National Natural Science Foundation of China (81071963) to XW.

Please address correspondence to: Dr. Xian Wang, Biomedical Research Center, Sir Runrun Shaw Hospital, Zhejiang University, Hangzhou, China, Tel: +86 571 86006927; Fax: +86 571 86006145; Email: wangx118@yahoo.com; Dr. Hongchuan Jin, Biomedical Research Center, Sir Runrun Shaw Hospital, Zhejiang University, Hangzhou, China, Tel: +86 571 86006366; Fax: +86 571 86006145; Email: jinhc@srsh.com

References

MicroRNA-204 downregulation in gastric cancer


