Abnormal expression of TRIB3 in colorectal cancer: a novel marker for prognosis

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BACKGROUND: TRIB3 is a human homologue of Drosophila tribbles. Previous studies have shown that TRIB3 controls the cell growth through ubiquitination-dependent degradation of other proteins, whereas its significance in the prognosis of colorectal cancer (CRC) is not yet fully understood.

MATERIALS: This study comprised 202 patients who underwent surgery for CRC, as well as 22 cell lines derived from human gastrointestinal cancer. The correlation of gene expression with clinical parameters in patients was assessed. The biological significance was evaluated by knockdown experiments in seven colorectal cancer cell lines.

RESULTS: A total of 20 cancer cell lines (90.9%) expressed the TRIB3 gene. The assessment in surgical specimens indicated that the gene expression was significantly higher in the cancerous region than in the marginal non-cancerous region. Patients with high TRIB3 expression were statistically susceptible to a recurrence of the disease, and showed poorer overall survival than those with low expression. The assessment of TRIB3 knockdown in five cell lines showed that small interfering RNA (siRNA) inhibition resulted in a statistically significant reduction in cell growth.

CONCLUSION: These data strongly suggest the usefulness of TRIB3 as a marker for predicting the prognosis of CRC patients, showing a basis for the development of effective treatments for CRC.

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In many developed countries, including the United States and Japan, cancer is one of the most prominent illnesses in public welfare and health measures (Jones et al, 2007; Jemal et al, 2008). The incidence of colorectal cancer (CRC) has increased significantly in recent years, in concert with the changing lifestyle (Kohno et al, 2007). The major cause of death in CRC is liver metastases (Yamasaki et al, 2007). Although treatment of CRC has improved recently, it fails in approximately one-third of the patients who need an alternative strategy for coping with death (Jones et al, 2007). In this matter, useful predictive markers would be desired in the medication of CRC patients.

As shown in other tumours, tumour-promoting oncogenes and tumour suppressors control cell proliferation through cell-cycle arrest of CRC (Aliaga et al, 1999; Jemal et al, 2008; Yamatodani et al, 2009). Further identification of genes responsible for the development and progression of CRC, as well as understanding of their clinical significance, would lead to efficient diagnosis and treatment of the disease. Characterization of key molecules is particularly promising for the development of new approaches for the treatment of gastrointestinal tumours.

Previous studies have shown that chromosomal aberrations occur during carcinogenesis, and relate to patients’ prognoses in CRC (Hermsen et al, 2002; Leslie et al, 2003). Alterations of particular loci at chromosome 20 are specifically associated with mutations in the tumour suppressor gene, TP53, by a survey of 50 cases of CRC, and they are also correlated with the progression of CRC, suggesting that the tumour suppressor pathway is involved in the maintenance of particular chromosomal regions (Wang et al, 2001; Pledger et al, 2005; Yde et al, 2007; Goodwin et al, 2008; Shor et al, 2008). It has been shown that aberrant gains at chromosome 20 are specifically associated with mutations in the tumour suppressor gene, TP53, by a survey of 50 cases of CRC, and they are also correlated with the progression of CRC, suggesting that the tumour suppressor pathway is involved in the maintenance of particular chromosomal regions (Wang et al, 2001; Leslie et al, 2003; Pledger et al, 2005; Yde et al, 2007; Goodwin et al, 2008; Shor et al, 2008).

Although previous studies suggest candidate genes in the regions at chromosome 20, which might have a role in CRC, it is yet to be fully understood in prognostic value (Wu et al, 2006; Zheng et al, 2008; Antonacopoulou et al, 2008). Here we report on TRIB3 gene in the chromosomal region at 20p13, which is overexpressed in CRC, as a new marker for prognosis and metastatic metastasis. Trib3 is a human homologue of Drosophila tribbles 3, which regulates cell growth, differentiation, oogenesis and metabolism by promoting ubiquitination-dependent degradation of other proteins, interacts with several transcriptional factors and is expressed in several tumours (Mata et al, 2000; Bowers et al,
2003; Du et al, 2003; Koo et al, 2004; Boudeau et al, 2006; He et al, 2006; Koh et al, 2006; Matsushima et al, 2006; Ord et al, 2007; Kato and Du, 2007; Xu et al, 2007; Yao and Nyomba, 2008). We studied the TRIB3 gene in 202 paired cancerous and non-cancerous regions of CRC, as well as 7 colorectal cancer cell lines and 15 other gastrointestinal cancer cell lines. Our data indicate the clinical significance of TRIB3 in the evaluation of CRC prognosis.

MATERIALS AND METHODS

Cell lines and culture

A total of 22 cell lines derived from human CRC and other gastrointestinal cancer (for CRC: Caco2, DLD-1, LoVo, HCT116, HT-29, KM12SM and SW480; for oesophageal cancer: TE-5, TE-8 and TE-10; for gastric cancer: MKN28 and MKN45; for pancreatic cancer: MiaPaCa-2, Panc-1 and PSN-1; for hepatocellular carcinoma: HuH-7, HepG2, Hep3B, HLE, HLF and PLC; for cholangiocellular carcinoma: HuCCT-1) were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and antibiotics at 37°C in a 5% humidified CO2 atmosphere. For small interfering RNA (siRNA) inhibition, double-stranded RNA duplexes targeting human TRIB3 (5'-GGGGUGGAGGAGGUGGAGCAACACUUA-3' and 5'-GGGUAGUCCUAGCCGUGCGCUUU-3') were purchased as a Validated Stealth RNAi kit (Invitrogen, Carlsbad, CA, USA), as well as negative control siRNA (12935-112, Stealth RNAi Negative Control, Medium GC Duplex, Invitrogen). CRC cell lines were transfected with siRNA at a concentration of 20 μmol ml⁻¹ using lipofectamine RNAiMAX (Invitrogen), incubated in glucose-free Opti-MEM (Invitrogen) and analysed using CellTac, a proliferation assay kit (Invitrogen). Values are presented as means ± s.d. from all independent experiments performed in triplicate.

Clinical tissue samples

The study comprised 202 patients who underwent surgery for CRC, including 118 patients at Kyusyu University from 1992 to 2002, and 84 patients at Osaka University from 2002 to 2006. Primary CRC specimens and adjacent normal colorectal mucosa were obtained from patients after written informed consent had been confirmed, in accordance with institutional ethics guidelines. The surgical specimens were fixed in formalin, processed through graded ethanol and embedded in paraffin, and were sectioned with a thickness of 4 μm. All sections were deparaffinized and stained with haematoxylin and eosin staining (see the Supplementary Information). Immunohistochemistry (IHC) was performed using a primary anti-Trib3 antibody complex was incubated overnight at 4°C. ENVISION reagents (Dako Cytomation, Glostrup, Denmark) were used to detect the signal from the antigen–antibody reaction. All sections were counterstained with haematoxylin. The primary anti-Trib3 rabbit polyclonal antibody (HPA015272; Sigma, St Louis, MO, USA) was used at a dilution of 1:100. All sections were independently examined for protein expression, and assessed by comparison of staining between normal and cancer regions under microscopic examination of ≥100 fields in each specimen.

Proliferation assay

To determine the proliferative properties, 1.0 × 10⁵ cells were seeded and cultured into each 24-well dish. The cell growth rate was measured by counting cells using a CellTac kit (Nihon Koden, Tokyo, Japan).

Statistical analysis

For continuous variables, data are expressed as mean ± s.d. The relationship between TRIB3 expression and clinico-pathological factors was analysed using χ² and Student’s t-tests. Kaplan–Meier survival curves were plotted and compared with the generalised log-rank test. Univariate and multivariate analyses for the identification of prognostic factors were performed using a Cox proportional hazard regression model. All tests were analysed using JMP software (SAS Institute, Cary, NC, USA). Differences with P-values < 0.05 were considered statistically significant.

RESULTS

Expression of TRIB3 in CRC cell lines and clinical tissue specimens

We first studied the expression of TRIB3 gene, and evaluated it in gastrointestinal cancer cell lines and clinical tissue samples.
by RT–PCR analysis to confirm that the PCR amplification was specific and produced a single band in agarose gel, stained with ethidium bromide, before performing real-time PCR. The RT–PCR study of TRIB3 in 22 human gastrointestinal cancer lines indicated 20 cells (90.9%; TE-8, TE-10, MKN45, MiaPaCa-2, Panc-1, PSN-1, Huh7, HepG2, Hep3B, HEL, HLF, PLC, HuCCT-1, Caco2, DLD-1, LoVo, HCT116, HT-29, KM12SM and SW480) that expressed the TRIB3 gene with a band in gel (the Supplementary Figure S1A). The RT–PCR analysis of TRIB3 in primary CRC samples was then performed in paired normal and tumour samples (representative data shown in Supplementary Figure S1B: TRIB3 expression was higher in cancerous regions than in normal regions). Quantitative real-time RT–PCR on 202 paired cancer and normal samples showed that 181 of 202 (89.6%) samples had higher levels of TRIB3 mRNA in cancerous regions than in paired normal regions. The mean expression value of TRIB3 mRNA in tumour regions, 154.62 ± 1021.63 (mean ± s.d.; normalised by GAPDH gene expression), was significantly higher than the value, 6.98 ± 4.91, for the corresponding normal regions ($P<0.001$; Student’s $t$-test). GAPDH = glyceraldehydes-3-phosphate dehydrogenase; RT–PCR = reverse transcriptase PCR; TRIB3 = tribbles homologue 3.

Expression of Trib3 protein

Figure 2 shows a representative immunohistochemical staining pattern for Trib3 in tissue from a CRC patient. Trib3 protein staining was observed in the nucleus and cytoplasm in epithelial cells; the expression of CRC was compared with non-cancerous epithelial cells, whereas the expression was appreciably weak or hardly detectable in stromal cells. Examination of 20 cases, which were selected randomly, indicated that 16 cases showed a higher expression level of Trib3 protein in cancerous regions compared with normal regions, whereas the remaining four cases showed no difference between normal and cancerous regions. To compare the data, mRNA expression was assessed by gel RT–PCR and real-time RT–PCR. The data show that mRNA expression was high level in all 16 immunohistochemistry-positive tumours, whereas mRNA expression was comparable in normal and cancerous regions of the remaining four tumours, suggesting that the high expression of Trib3 protein is associated with mRNA expression ($P<0.001$; $\chi^2$ test). No variation of staining intensity for Trib3 was observed in each of the specimens. We concluded that both mRNA and the protein coded by this gene are associated and frequently expressed together in CRC.
To study the TRIB3 expression in CRC quantitatively, the data were classified into two experimental groups on the basis of the TRIB3 expression levels to assess the expression value without any bias. The high-expression group comprised patients who had a level of TRIB3 expression higher than the median value for TRIB3/GAPDH expression in tumour regions compared with normal.

**Table 2** Univariate and multivariate analysis for overall survival (Cox proportional hazards regression model)

| Factors                      | Univariate analysis | Multivariate analysis |
|------------------------------|---------------------|-----------------------|
| Age (years)                  |                     |                       |
| (< 67/67 <)                  | 1.23                | 0.85 – 1.80           | 0.258 |
| Gender                       | 1.93                | 0.90 – 4.47           | 0.090 |
| Histological grade           | 1.54                | 0.36 – 4.35           | 0.511 |
| Tumour size                  | 3.70                | 1.69 – 15.66          | 0.001 |
| Tumour invasion              | 11.00               | 3.28 – 68.37          | <0.001 |
| Lymph node metastasis        | 4.28                | 2.02 – 9.63           | 0.001 |
| Lymphatic invasion           | 2.44                | 1.14 – 5.44           | 0.021 |
| Venous invasion              | 2.17                | 0.92 – 4.73           | 0.071 |
| Metastasis                   | 21.89               | 9.33 – 60.11          | <0.001 |

TRIB3 mRNA expression

| Median ＜ / ≤ median | Univariate analysis | Multivariate analysis |
|---------------------|---------------------|-----------------------|
| 8.45                | 2.97 – 35.48        | <0.001 |
| 3.70                | 1.27 – 16.35        | 0.014 |

RR = relative risk; CI = confidence interval; Wel = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocarcinoma and mucinous carcinoma; TRIB3 tribbles homologue 3.

The statistic significance is shown with under lines.

**TRIB3 expression and clinico-pathological characteristics**

To study the TRIB3 expression in CRC quantitatively, the data were classified into two experimental groups on the basis of the TRIB3 expression levels to assess the expression value without any bias. The high-expression group comprised patients who had a level of TRIB3 expression higher than the median value for TRIB3/GAPDH expression in tumour regions compared with normal.

**Table 3** Univariate and multivariate analysis for metachronous metastasis-free over 5 years survival rate (Cox proportional hazards regression model)

| Factors                      | Univariate analysis | Multivariate analysis |
|------------------------------|---------------------|-----------------------|
| Age (years)                  |                     |                       |
| (< 67/67 <)                  | 1.33                | 0.85 – 2.09           | 0.020 |
| Gender                       | 2.44                | 0.97 – 6.90           | 0.055 |
| Histological grade           |                     |                       |
| (Wel-Mod / others)           | 24.0                | 4.78 – 101.61         | <0.001 |
| Tumour size                  | 3.66                | 1.66 – 15.55          | <0.001 |
| Tumour invasion              | 4.80                | 1.61 – 20.58          | 0.003 |
| Lymph node metastasis        | 4.01                | 1.65 – 10.26          | 0.002 |
| Lymphatic invasion           | 4.49                | 1.73 – 13.83          | 0.001 |
| Venous invasion              | 3.10                | 1.21 – 7.53           | 0.019 |

TRIB3 mRNA expression

| Median ＜ / ≤ median | Univariate analysis | Multivariate analysis |
|---------------------|---------------------|-----------------------|
| 4.33                | 1.45 – 18.59        | <0.006 |
| 3.86                | 1.09 – 19.00        | 0.035 |

RR = relative risk; CI = confidence interval; Wel = well differentiated adenocarcinoma; Mod = moderately differentiated adenocarcinoma; Others = poorly differentiated adenocarcinoma and mucinous carcinoma; TRIB3 tribbles homologue 3.

The statistic significance is shown with under lines.
After the primary operation, the median follow-up was 6.31 years. We evaluated the metachronous, metastasis-free survival over 5 years in these patients, indicating that the rate was significantly lower in patients of the high-expression group \( (P = 0.007, \text{Figure 4}) \). Table 3 shows the univariate and multivariate analyses of factors related to patient prognosis. Univariate analysis showed that the post-operative metastasis was significantly correlated with following factors: histological grade \( (P < 0.001) \), tumour size \( (P < 0.001) \), tumour invasion \( (P = 0.003) \), lymph node metastasis \( (P = 0.001) \), lymphatic invasion \( (P = 0.001) \), venous invasion \( (P = 0.019) \) and \( TRIB3 \) mRNA expression \( (P = 0.006) \). Multivariate regression analysis indicated that inclusion in the \( TRIB3 \) high-expression group \( (RR = 3.86; 95\% \ CI = 1.09–19.00; P = 0.035) \) was an independent predictor of metastasis-free survival, as were histological grade \( (RR = 25.9; 95\% \ CI = 3.57–215.84; P = 0.001) \) and tumour size \( (RR = 3.04; 95\% \ CI = 1.18–13.62; P = 0.017) \).
DISCUSSION

This study showed that TRIB3 is expressed at higher levels in CRC than in the corresponding normal regions, and is expressed in gastrointestinal cancer cell lines. The siRNA inhibition experiment showed the functional relevance of expressed TRIB3 in gastrointestinal cancer cell lines. To the best of our knowledge, this study is the first to show the candidacy of TRIB3 as a prognostic CRC marker, supported by the functional relevance to cell growth.

Nowadays, it can be useful to determine the necessity of intensive follow-up and adjuvant therapy for CRC by predicting recurrence and metastases in curative surgical resection (Bathe et al., 2004; Kornmann et al., 2008; Wolpin and Mayer, 2008). In this study, clinico-pathological analysis revealed that TRIB3 is closely related to metastasis, but not to lymphatic metastasis. It may correlate with some mechanism of little concern to invasiveness. Patients with CRCs with high TRIB3 expression showed a poorer prognosis for disease-free and overall survival than those in the low-expression group. Data indicate that TRIB3 is an independent prognostic factor, as well as a very important predictor that is already known (Derkinderen et al., 1990). TRIB3 is presumably a good predictor of metachronous metastasis that can be followed by curative surgical intervention. In gastrointestinal cancer therapy, it is essential to prevent metachronous metastasis. Several adjuvant chemotherapies are helpful in certain disease stages, especially in CRC (Bathe et al., 2004; Andre et al., 2007). Recently, increasing evidence has been accumulated, showing the usefulness of less invasive surgery in the treatment of CRC, such as laparoscopic and endoscopic surgery (Lacy et al., 2002; Weeks et al., 2002; Clinical Outcomes of Surgical Therapy Study Group, 2004; Jane et al., 2007). For these cases, predictive markers of tumour invasion and metastasis, which are independent of traditional TNM classification and contribute collectively to diagnoses and treatments, are very important. These data indicate the candidacy of TRIB3.

Although improving treatments such as pre-operative and post-operative chemotherapy and radiotherapy combined with surgery for CRC have contributed to the reduction of recurrences and metastases, half of the cases eventually metastasise despite systemic chemotherapy followed by surgery (Koshariya et al., 2007). Adjuvant chemotherapy for CRC has been desirable in highly suspicious metastatic cases. In these cases, the assessment of TRIB3 expression may be useful to predict patient prognosis.

In biological assessment, this study showed that TRIB3 expression was related to tumour growth in several gastrointestinal cancer cell lines. The in vivo study showed that siRNA inhibition of TRIB3 resulted in a reduction in cell growth of seven gastrointestinal cancer cell lines, significantly ($p < 0.05$). Although previous reports showed that TRIB3 is expressed in several cancer cell lines, this study shows that TRIB3 seems to stimulate proliferation, and may be a new target for the therapy of gastrointestinal cancer (Bowers et al., 2003; Xu et al., 2007).

TRIBs, belong to the pseudokinase family consisting of three mammalian isoforms, TRib1, TRib2 and TRib3, have no detectable kinase enzymatic activity because of variations in key amino acids in the ATP-binding domain, but possess substrate-binding domains relating to their function as protein-interacting modules (Seher and Leptin, 2000; Yamatodani et al., 2009). TRIBs associate with large proteins such as transcriptional factors, and regulate cell growth, differentiation and metabolism (Bouneau et al., 2006).

TRib1 interacts with Mapk and modulates Mapk activity associated with smooth muscle cell proliferation and migration (Kiss-Toth et al., 2004; Sung et al., 2007). TRib2 has a role in adipogenesis in combination with the degradation of C/EBPbeta (Naik et al., 2007). TRib3 promotes ubiquitination and degradation of proteins involved in cell-cycle regulation and oogenesis through an interaction with activation transcription factor 4, and is involved in the Pten pathway through interaction with Akt (Mata et al., 2000; Du et al., 2003; He et al., 2006; Koh et al., 2006; Kato and Du, 2007; Yao and Nyomba, 2008). TRib3 expression is increased in several primary tumours and cancer cell lines and can be controlled by nutrient starvation, which is consistent with these data (Bowers et al., 2003; Schwarzer et al., 2006; Xu et al., 2007). Our report indicates that TRIB3 is not only a new independent prognostic factor and predictor of metachronous metastasis, but is also a useful target because the inhibition of TRIB3 may lead to the reduction of CRC through the control of cell growth.

Supplementary Information accompanies the paper on British Journal of Cancer website (http://www.nature.com/bjc)

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