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Focal Adhesion Plaque Associated Cytoskeletons Are Involved in the Invasion and Metastasis of Human Colorectal Carcinoma

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ABSTRACT
The protein and mRNA expression of focal adhesion plaque associated cytoskeletons, including talin, vinculin, paxillin, and tensin, was studied using immunofluorescence in combination with confocal laser scanning microscopy and fluorescent quantitative polymerase chain reaction in 41 matched samples of human normal colorectal mucosa, primary colorectal adenocarcinomas, and 19 separate lymph node metastases. All specimens showed expression. The results showed talin, vinculin, tensin, and paxillin expression were correlated with carcinogenesis, invasion, and metastasis of colorectal carcinoma (CRC). Talin, vinculin, and tensin underwent downregulation while paxillin went up. So these cytoskeletons may play bidirectional regulating roles during the progression of CRC.

INTRODUCTION
Invasion into surrounding tissues is a prominent phenotype of cancer cells (1, 2). Regulatory mechanism of cell motility is undoubtedly critical in this process. The generation of motile force through the organization of actin cytoskeletons and the formation of focal adhesion plaque (FAP) is necessary for the motility of tumor cells (3). The FAP is an important linker between actin cytoskeleton and plasma membrane, which consists of a complex of proteins that assemble at sites where the cancer cells are attached at the extracellular matrix (ECM). A number of proteins, such as paxillin, vinculin, talin, and tensin, have been found to be preferentially associated with the focal adhesion complex. They are also named as cytoskeletons which are involved in signaling events between cells and their ECM and function in cellular motility (2), integrin-signaling pathways (3–6), and apoptosis (7–9). The interaction between integrins and their ligands promotes the formation of FAP. In other words, FAP is the structural basis for integrin signaling.

FAP-associated cytoskeletons can be classified into two groups: one is the structural protein including vinculin, talin, and tensin, and the other is the regulatory protein including paxillin etc. (10–12). However, relatively little is known about the differential expression of various FAP-associated cytoskeletons between metastatic nodules and primary lesions. Recently, several previous studies on the relationship between integrin signal transduction and the invasion and metastasis of colorectal carcinoma (CRC) have been conducted with cultured cells, but they failed to reflect the actual biological properties of tumor cells in vivo and quantify the protein and mRNA expression of cytoskeletons (13–18).

In the present study, we compared mRNA and protein expression levels of four FAP-associated cytoskeletons (paxillin, vinculin, talin, and tensin) in matched samples of human normal colorectal mucosa, primary colorectal adenocarcinomas, and lymph node metastases, providing a direct insight into the expression status of different focal adhesion proteins with the same genetic background. Fluorescent quantitative polymerase chain reaction (FQ-PCR) and immunofluorescence in
combination with confocal laser scanning microscopy (CLSM) were applied. The roles of FAP-associated cytoskeletal proteins during the invasion and metastasis of CRC were evaluated.

**MATERIALS AND METHODS**

**Detection of protein expression of FAP-associated cytoskeletons using CLSM**

**Tissue samples**

Forty-one matched tissue samples of primary colorectal adenocarcinomas (T), adjacent normal colorectal mucosae (N), and 19 separate lymph node metastases (M) were collected from patients undergoing surgical resection in Nanfang Hospital of Guangdong, China. Adjacent N specimens were obtained from the sites 15 cm apart from the primary tumors. Tissue samples were separated from necrotic tissues by sharp dissection. Histopathological confirmation was done by a reference pathologist. Grading and staging of tumor were determined according to WHO criteria. Half of the tissues were snap-frozen in liquid nitrogen and then stored at −80°C for FQ-PCR analysis, and another half were formalin-fixed and paraffin-embedded before serial 4-μm sections cut from each paraffin block were placed on poly-l-lysine-coated slides and then stored at 4°C for CLSM analysis.

**Antibodies**

Antibodies including mouse monoclonal anti-talin (clone TA205; Labvision Co., Fremont, CA, USA), mouse monoclonal anti-paxillin (clone 5H11; Labvision Co.), mouse monoclonal anti-vinculin (clone VLN01; Labvision Co.), and mouse monoclonal anti-tensin (clone 5; Pharmingen Co., San Diego, CA, USA) were used for CLSM analysis. They were diluted as follows: mouse monoclonal anti-talin antibody (1:50), anti-paxillin antibody (1:100), anti-vinculin antibody (1:50), and anti-tensin antibody (1:50).

**Immunofluorescent staining**

Immunofluorescent staining using SABC-FITC kit (Boster Biotechnology Co., Wuhan, China) was performed according to manufacturer recommendation. Four-micrometer sections from formalin-fixed, paraffin-embedded tissues were mounted on poly-l-lysine-coated slides. They were air-dried, deparaffinized, and then rehydrated and washed with phosphate-buffered saline (PBS). After nonspecific binding sites of the sections were blocked by treatment with 2% normal goat serum in PBS for 30 min at room temperature, all sections were incubated overnight at 4°C with the mouse monoclonal antibodies listed above. The sections were rinsed with PBS and incubated with biotinylated goat antimouse IgG for 30 min at room temperature followed by washing with PBS. The sections were then incubated with streptavidin-fluorescein isothiocyanate for 20 min in a dark chamber followed by washing extensively with PBS. The nucleus was stained with propidium iodide (5 μg/mL; Sigma Co., St. Louis, MO, USA) for 30 min in a dark chamber followed by washing extensively with PBS. Coverslips were mounted with aqueous mounting medium or 90% glycerol in PBS. Slides were stored in the dark at room temperature (semipermanent mount) or at 4°C (glycerol/PBS mount). Positive and negative controls were included in each run. Finally, the sections were examined using CLSM and the protein expression levels were estimated quantitatively.

**Quantitative image analysis of FAP-associated cytoskeletons by CLSM**

The parameters of the CLSM (TCS-SP2 A0BS; Leica, Germany) were set as follows: Scan Mode: XYZ; Format: 512 × 512; Sections: 1; AV Line: 6; Gain PMT1: 677 V (talin, vinculin) and 657 V (paxillin, tensin); Gain PMT2: 647 V; Beam Exp: 1; Pinhole: 1.00 Airy; Zoom: 1.00; Objective: HCX PL APO 100×, Oil.

Confocal imaging analyses were performed using the 488-nm excitation laser line and simultaneous dual display mode (518-nm emission and phase contrast) of the BioRad Laser Sharp Imaging program. Five random images were collected to determine the average fluorescence intensity representing protein expression levels.

**Detection of mRNA expression of FAP-associated cytoskeletons using FQ-PCR**

**Synthesis of FAP-associated cytoskeleton-specific probes**

Nonextendable oligonucleotide probes specific for talin, vinculin, paxillin, and tensin were designed respectively according to cDNA sequences from GenBank using the Primer express 2.0 (Applied Biosystem, Inc.). These probes were labeled with 3′TAMRA (6-carboxy-tetramethylrhodamine) which was combined with a minor groove binder (MGB) and 5′FAM (6-carboxyfluorescein), and then purified by high-performance liquid chromatography (Integrated DNA Technologies, Coralville, IA, USA).

The sequences of the forward primers, reverse primers, and probes for talin, vinculin, paxillin, and tensin are summarized in Table 1.

**FQ-PCR conditions**

The probe was hybridized to the target cDNA between the 5′ and 3′ oligonucleotides and, as amplification proceeded, the 5′-fluorophor was cleaved off the probe by 5′ nuclease activity of the polymerase. Free in solution, the 5′-fluorophor was no longer quenched by TAMRA, resulting in increased fluorescence at 518 nm. Ribogreen (Molecular Probes, Eugene, OR, USA) fluorescence was used to accurately quantitate starting levels of 10 ng/μL total RNA extracted from the tumor samples. Fluorescence intensity produced during the PCR amplifications in each of 96 wells was monitored every 8.5 s using the 96-well thermal cycler ABI PRISM 7000 (Perkin-Elmer-ABI, Foster City, CA, USA). A real-time amplification plot was
generated for each well, for which the number of amplification cycles was plotted on the x-axis and the log of change in fluorescence over baseline (ΔRn = fluorescence-baseline fluorescence) on the y-axis. The instrument’s software calculated a threshold cycle number (Ct) at which each PCR amplification reached a significant threshold level. This threshold cycle was directly proportional to the number of specific template copies present in the sample. Positive control and absolute standard were used in all assays. Using this absolute standard, the message levels were measured accurately over the five-log range from 200 copies to 90 million template copies (0.1 femtograms to 2 × 10^5 femtograms).

The amplification conditions were 40 cycles of denaturation at 93°C for 1 min, annealing at 55°C for 1 min, with an initial 2 min of extra denaturation at 93°C.

RNA isolation and quantitation of mRNA expression of FAP-associated cytoskeletons

Total RNA was isolated using the guanidinium isothiocyanate-based system (Rneasy; Qiagen, Valencia, CA, USA). Snap-frozen tumor specimens were poweder-homogenized (Power Gen 125; Fisher Scientific, Pittsburgh, PA, USA) before chloroform extraction. Any DNA contamination was resolved by treating total-RNA isolates with DNase (RNasefree; Ambion, Austin, TX, USA). Each mRNA unknow was tested for DNA contamination by including DNase (RNasefree; Ambion, Austin, TX, USA). Each mRNA unknow was tested for DNA contamination by including DNase (RNasefree; Ambion, Austin, TX, USA). Each mRNA unknow was tested for DNA contamination by including DNase (RNasefree; Ambion, Austin, TX, USA). Each mRNA unknow was tested for DNA contamination by including DNase (RNasefree; Ambion, Austin, TX, USA). Each mRNA unknow was tested for DNA contamination by including DNase (RNasefree; Ambion, Austin, TX, USA).

All of the CRCs and lymph node metastases were analyzed for expression levels of talin, vinculin, paxillin, or tensin in individual experiments using the same master reaction mix to minimize experimental variability. Each tissue sample was tested in triplicate and the mean femtogram expression level was calculated and converted to copy number using the formula (6.02 × 10^23 copies/mol) × (measured grams of talin, vinculin, paxillin, or tensin)/(molecular weight of talin, vinculin, paxillin, or tensin message).

Statistical methods

All samples were grouped according to their tissue type, infiltrating depth, and lymph node metastasis. Data were analyzed by nonparametric test using SPSS 10.0 (SPSS Inc., Chicago, IL, USA) and a p value less than .05 was considered statistically significant.

RESULTS

CLSM analysis of talin, vinculin, paxillin, and tensin in human colorectal adenocarcinomas

To examine the expression of FAP-associated cytoskeletons at the histological level, we performed CLSM analysis using the four monoclonal antibodies described above. The protein expression levels (represented by fluorescence intensity) of four FAP-associated cytoskeletons in matched samples of human normal colorectal mucosae (N), primary colorectal adenocarcinomas (T), and lymph node metastases (M) were compared. Table 2 shows the protein expression levels of talin, vinculin, paxillin, and tensin in N, T, and M from 41 matched cases (Figure 1).

As shown in Table 2, the level of paxillin protein expression increased gradually during the transition from normal colorectal mucosae to malignant lesions and metastases. The expression level of paxillin in CRCs with lymph node metastasis was significantly higher than that of those without lymph node metastasis. The paxillin expression was positively correlated with the infiltrating depth of carcinoma.

Unlike paxillin, the expression levels of talin, vinculin, and tensin in CRCs were significantly lower than those in normal colorectal mucosae. Compared with primary CRCs, lymph node metastases showed even lower expression levels of talin, vinculin, and tensin. In addition, CRCs with lymph node metastasis had less expression of talin, vinculin, and tensin than those without lymph node metastasis. The deeper carcinomas infiltrated, the less talin, vinculin, and tensin were expressed.

FQ-PCR analysis of talin, vinculin, paxillin, and tensin in CRC

Subsequently, we extended our observations to mRNA level using FQ-PCR analysis to further validate the result of CLSM analysis (Figure 2). We examined the mRNA expression of paxillin in 41 matched samples of normal colorectal mucosae and primary CRC (Table 3) and found that paxillin expression was significantly higher in CRCs than in matched normal colorectal mucosae. In addition, the expression level of paxillin in CRCs with lymph node metastasis was significantly higher than that of those without lymph node metastasis. The paxillin expression was positively correlated with the infiltrating depth of carcinomas. In contrast to paxillin expression, the expression levels of talin, vinculin, and tensin in CRCs were significantly lower than those in normal colorectal mucosae. CRCs with
Table 2. Protein expression of talin, vinculin, tensin, and paxillin in matched samples of normal colorectal mucosae (N) and primary colorectal cancer (T) as well as in a separate set of colorectal lymph node metastasis (M).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Talin</th>
<th>Vinculin</th>
<th>Tensin</th>
<th>Paxillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Median</td>
<td>Median</td>
<td>Median</td>
</tr>
<tr>
<td>Tissue type</td>
<td>n</td>
<td>p</td>
<td>p</td>
<td>p</td>
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<tr>
<td>T</td>
<td>41</td>
<td>77.26</td>
<td>78.96</td>
<td>55.00</td>
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<tr>
<td>M</td>
<td>19</td>
<td>64.39 .000^a</td>
<td>65.53 .000^a</td>
<td>47.56 .018^a</td>
</tr>
<tr>
<td>N</td>
<td>41</td>
<td>116.78 .000^a</td>
<td>118.82 .000^a</td>
<td>97.67 .000^a</td>
</tr>
<tr>
<td>Infiltrating depth</td>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>90.94</td>
<td>86.47</td>
<td>57.20</td>
</tr>
<tr>
<td>Muscularis or serosa</td>
<td>26</td>
<td>70.19 .002</td>
<td>72.09 .003</td>
<td>49.42 .040</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>19</td>
<td>69.98</td>
<td>71.56</td>
<td>47.38</td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>92.66 .000</td>
<td>92.36 .000</td>
<td>61.40 .002</td>
</tr>
</tbody>
</table>

^aM or N compared with T.

Figure 1. Representative examples of CLSM image for talin, vinculin, tensin, and paxillin (fluorescein isothiocyanate for target proteins and propidium iodide for nucleus, × 1000). The talin, vinculin, and tensin immunoreactivity was intense in normal colorectal mucosae, moderate in primary adenocarcinoma, and weak in lymph node metastasis. Otherwise, the paxillin immunoreactivity was weak in normal colorectal mucosae, moderate in primary adenocarcinoma, and intense in lymph node metastasis. N, normal colorectal mucosae; T, primary colorectal adenocarcinoma; M, lymph node metastasis.
Figure 2. Fluorescent quantitative amplification plots and standard curves of absolute standard for talin, vinculin, tensin and paxillin. Delta Rn = fluorescence-baseline fluorescence; C_t, threshold cycle number; log CO, log value of original concentration of template.
lymph node metastasis had less expression of talin, vinculin, and tensin than those without lymph node metastasis. The deeper carcinomas infiltrated, the less talin, vinculin, and tensin were expressed (Table 3).

### DISCUSSION

Histopathological confirmation of surgical specimens used in CLSM and FQ-PCR analysis revealed that primary colorectal adenocarcinoma specimens consisted of adenocarcinoma cells, stromal cells, and normal mucosal tissues, while normal mucosal specimens belonged to nontumorous mucosal tissue.

Several previous studies on the relationship between integrin signal transduction and the invasion and metastasis of CRC have been conducted by cultured cells, but they failed to reflect the actual biological properties of cancer cells in vivo, and did not quantify the protein and mRNA expressions of cytoskeletons (13–18).

It is known that regulatory mechanism of cell motility is critical in the process of cancer invasion which is a prominent phenotype of cancer cells (1, 2). The generation of motile force through the organization of actin cytoskeletons and the formation of FAP is necessary for the motility of tumor cells (3). The FAP, consisting of a complex of proteins, is an important linker between actin cytoskeleton and plasma membrane. Quite a few proteins are found to be preferentially associated with the focal adhesion complex including paxillin, vinculin, talin, and tensin, etc. (19–22). These proteins, also named as cytoskeletons, are involved in signaling events between cells and their ECM. The link between integrins and their ligands promotes the formation of FAP which is the structural basis for integrin signaling. FAP-associated cytoskeletons are classified into two groups: one is the structural protein including vinculin, talin, and tensin, and the other is the regulatory protein including paxillin. However, till date, relatively little is known about the expression levels of FAP-associated cytoskeletons in metastatic nodules compared with primary lesions.

Paxillin is a unique focal adhesion protein with its ability to interact with certain oncogene products (23). Paxillin also functions as part of signaling proteins. Vadlamudi et al. (24) showed that heregulin stimulation of noninvasive human breast cancer MCF-7 cells resulted in the upregulation of paxillin expression, leading to the development of invasive phenotype. In contrast, Salgia et al. (25) reported that the transfection of human non-small cell lung cancer cells with paxillin cDNA consistently reduced cell motility. In the present study, the average paxillin expression level was significantly higher in T than in N, and in M than in T (Tables 2 and 3). The expression level of paxillin in CRCs with lymph node metastasis was significantly higher than that in CRCs without lymph node metastasis. The paxillin expression was positively correlated with the infiltrating depth of carcinoma. These results demonstrate that the level of paxillin expression may increase gradually during the transition from normal colorectal mucosae to malignant lesions and metastases, suggesting that paxillin may promote carcinogenesis, invasion, and metastasis of CRC.

Vinculin, talin, and tensin are actin-membrane attachment proteins that exist at the intracellular surface of the plasma membrane as FAPs intervening between F-actin and integrins (26–30). Sadano et al. (31) showed through Northern blot and immunohistochemical analyses that the expression of vinculin was lower in weakly metastatic mouse B16-melanoma cells than in highly metastatic counterparts. Lifschitz-Mercer et al. (32) reported that the vinculin expression was downregulated in human metastatic squamous cell carcinomas compared with the expression in nonmetastatic carcinoma cells. In the present study, the average expression levels of talin, vinculin, and tensin in T were significantly lower than those in N. Compared with the primary CRCs, the lymph node metastases showed even lower expression levels of talin, vinculin, and tensin. CRCs with lymph node metastasis had less expression of talin, vinculin, and tensin than those without lymph node metastasis. The deeper the carcinomas infiltrated, the less the talin, vinculin, and tensin were expressed (Tables 2 and 3). These results indicate that the levels of talin, vinculin, and tensin expression may decrease gradually during the transition from normal colorectal mucosae to malignant lesions and metastases, suggesting that talin, vinculin, and tensin may inhibit the carcinogenesis, invasion, and metastasis of CRC, somewhat similar to the tumor suppressor genes.

Evidences for reduced apoptosis in metastatic colorectal tumors have been presented by Hashimoto et al. (33), who
examined intranuclear DNA strand breaks localized with in situ nick translation to measure the frequency of apoptosis in human colorectal cancer specimens. They found that the labeling indices of carcinomas that did metastasize to lymph nodes or the liver were lower than that of carcinomas that did not metastasize. Thus, liver metastases were more likely to have less apoptotic cells (33). These findings support the conceptual framework whereby tumor cells that become invasive and metastatic are subjected to intense apoptotic pressure. This would select for cells that are resistant to apoptosis so that they could successfully grow and become sites of distant metastasis. Thus, our finding that paxillin expression is upregulated and talin, vinculin, and tensin expression are downregulated in colorectal lymph node metastases fits into this framework; enhanced survival signaling through FAP may promote continued growth of the metastatic colorectal tumor cells. Furthermore, our findings are consistent with the opinion that adenocarcinoma cell motility may favor local dissemination from the primary cancer site and into circulation, whereas adenocarcinoma cells in the metastatic nodules may require downregulation of certain molecule expression.

In the present study, our correlation analysis showed that the protein expression of talin, vinculin, tensin, and paxillin was consistent with their mRNA expression (data not shown), providing evidences at both mRNA and protein expression levels that the FAP-associated cytoskeletons play important roles during the invasion and metastasis of CRC. Interestingly, FAP exerts bidirectional regulating roles during the development and progression of CRC. The structural proteins of FAP, including talin, vinculin, and tensin, were downregulated during the development and progression of CRC, whereas the regulatory protein of FAP, paxillin, was upregulated. Therefore, up- and downregulation of FAP-associated cytoskeleton expression observed in our clinical samples may have a profound effect on the process of development and progression of CRC. The associated mechanism remains to be further clarified.

Taken together, this study sheds light on the mechanisms of the invasion and metastasis of CRC. The evaluation of paxillin, talin, vinculin, and tensin, especially in primary CRC, may be useful in biological characterization of tumor cells with migratory ability, and may be helpful for clinical therapy which aims at integrin signal transduction in the near future.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


