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Mycoplasma infections and different human carcinomas

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Abstract

AIM To explore relationships between human carcinomas and mycoplasma infection.

METHODS Monoclonal antibody PD4, which specifically recognizes a distinct protein from mycoplasma hyorhinis, was used to detect mycoplasma infection in different paraffin embedded carcinoma tissues with immunohistochemistry. PCR was applied to amplify the mycoplasma DNA from the positive samples for confirming immunohistochemistry.

RESULTS Fifty of 90 cases (56%) of gastric carcinoma were positive for mycoplasma hyorhinis. In other gastric diseases, the mycoplasma infection ratio was 28% (18/49) in chronic superficial gastritis, 30% (14/46) in gastric ulcer and 37% (18/49) in intestinal metaplasia. The difference is significant with gastric cancer (χ^2 = 12.06, P<0. 05). In colon carcinoma, the mycoplasma infection ratio was 55.1% (32/58),but it was 20.9% (10/ 49) in adenomarous polyp (χ^2 = 13.46, P<0.005). Gastric and colon cancers with high differentiation had a higher mycoplasma infection ratio than those with low differentiation (P<0.05). Mycoplasma infection in esophageal cancer, lung cancer, breast cancer and glioma was 50.9% (27/53), 52.6% (31/ 59), 39.7% (25/63)and 41% (38/91), respectively. The mycoplasma DNA was successfully amplified with the DNA extracted from the cancer tissues that were positive for mycoplasma infection (detected with antibody PD4).

CONCLUSION There was high correlation between mycoplasma infection and different cancers, which suggests the possibility of an association between the two. The mechanism involved in oncogenesis by mycoplasma remains unknown.

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INTRODUCTION

Mycoplasma is one of the smallest living organisms isolated from nature, and can be cultured in a special medium. Mycoplasmas spread widely at the cell membrane of many types of mammaliane cells. Some can enter these cells[1-3]. As a conditional pathogenic organism, mycoplasmas have been associated with many diseases[4-11]. Experimental data indicates that some mycoplasmas cause chromosomal changes and cell transformations *in vitro* through progressive chromosomal loss and translocations[12-17], but the association between mycoplasmas and cancer remains unclear. Monoclonal antibody (MAb) PD4 was prepared with human gastric cancer cell line MGC803 as immunogen[18]. The antibody specifically reacted with an antigen associated with some tumor cell lines[19]. Western blot analyses indicated that molecular weight of this antigen (P40) was about 40 kilo-Daltons. Our previous study indicated that MAb PD4 could inhibit the growth of Rat 3-3 and GCM3T3 cells, as well as the tumorigenicity in nude mice[20]. Recently, the antigen P40 was identified with N-terminal sequence analysis. The 16 amino acids at the N-terminus of P40 are identical with the N-terminus of P37, which originated from Mycoplasma hyorhinis, and the PD4 reacted with mycoplasma hyorhinis strongly (unpublished). These results indicate that MAb PD4 is an antibody raised to mycoplasma, not to tumor cells. We then used PD4 detecting mycoplasma infections in paraffin-embedded gastrointestinal carcinoma tissues and other cancers. Here we report the detection results which show a strong association between mycoplasma infections and different human carcinomas.

MATERIALS AND METHODS

Specimen selection

The examined specimens were selected according to the results of hematoxylin-eosin (H&E) staining evaluated microscopically. There are 90 samples of

archived gastric carcinoma tissues and 44 samples of archived normal tissue around carcinoma for comparison, 67 samples are from men and 28 samples are from women. The pathologic grand I-II, II-III and III were 23, 18 and 49 cases, respectively, in the 90 tumor specimens. We selected 47 cases of superficial gastritis, 46 cases of gastric ulcer, and 49 cases of intestinal metaplasia to serve as controls. Fifty-eight cases of colon carcinoma and 49 cases of adenomarous polyp were chosen for detection. Besides the gastrointestinal samples, 53 cases of esophageal carcinoma, 59 cases of lung carcinoma, 63 cases of breast carcinoma, and 91 cases of glioma were also detected. Except the specimens of glioma, which were from 307th Hospital of the People's Liberation Army, all of the samples were kindly provided by the Department of Pathology of the First Affiliated Hospital of Jiangxi Medical College of China.

B-SA immunoperoxidase stain

Tissues embedded in paraffin were microtome-sectioned into 4 μm slices. The slices were floated onto a tissue flotation bath and mounted on 3-aminopropyltriethoxysilane (APES)-treated slides. Sections were heat-immobilized in an oven at 60℃ for 30 minutes, deparaffinized with three changes of xylene, sequentially dehydrated in different concentrations of ethanol, and rinsed in distilled water. Endogenous peroxidase activity was blocked by immersing sections in 0.3% H_2O_2 for 5-10 minutes, followed by sequential rinsing in distilled water and in phosphate buffered saline (PBS). The primary antibody, mouse anti-mycoplasma hyorhinis monoclonal antibody PD4 (described above prepared by our laboratory, diluted to 10 mg/L) was applied to the slices. Unspecialized mouse IgG was used as a negative control. After being incubated for 1 hr at 37℃ or overnight at 4℃, the slices were rinsed three times in PBS for 5 minute each. The slices were stained with B-SA kit (Biogenex) according to the manufacturer's instruction. The results were evaluated by two investigators independently. (-) means no yellow staining was observed in cells, (+) means some cells were observed to be lightly stained, but less than 50%, and (++) indicates that over 50% were observed to be stained, or the staining was strong.

Extraction of DNA from tissues embedded in paraffin

Human gastric cancer tissues embedded in paraffin were microtome-sectioned into 10 μm slices. Two or more slices from each sample were placed into individual sterile autoclaved microcentrifuge tubes. Sterility was maintained at all times and the microtome blade was cleaned completely with ethanol between sectioning to prevent cross-contamination. Digestion buffer, consisting of 0.5% Tween-20, 50mM Tris (pH 8.5), 1mM EDTA, and 200 mg/L proteinase K (Sigma Chemical Co), was added into each tube and incubated overnight. The DNA was successively extracted with equal volumes of Tris-saturated phenol (pH 8.0), 1:1 phenol/chloroform (vol/vol) and then chloroform. one/10 volume of 10M ammonium acetate was added to each sample and the DNA was precipitated by the addition of 2.5 volumes of 100% percent cold ethanol. The DNA was incubated at -20℃ overnight and centrifuged for 20 minutes at 4℃, 12 000 rpm. The DNA was washed with 70% ethanol, and suspended in distilled water.

Mycoplasma infection detected by PCR

Primers used to amplify conserved mycoplasma 16sr DNA were synthesized by Shengong Co. (Shanghai) and the sequences are as follows: forward primer: 5'-TACGGGAGGCAGCAGTA-3'; reverse primer: 5'-TCAAGATAAAGTCATT-3'. The PCR program consisted of 35 cycles at 94℃ for 30 seconds, 48℃ for 30 seconds, and 72℃ for 20 seconds, and 72℃ 10 minutes for final extension[21,22]. The PCR products were analyzed with agar gel electrophoresis.

Statistical analyses

The data were analyzed using the χ^2 test implemented in a commercially available computer program. A value of $P \leqslant 0.05$ was considered significant.

RESULTS

Mycoplasma infection in gastric carcinoma tissues and other gastric diseases

The results indicate that the gastric carcinoma tissues with high differentiation had a higher mycoplasma infection ratio than that of low differentiation gastric carcinoma tissues ($P<0.005$, Table 1). Seven samples exhibited different levels differentiation in the same specimen. The mycoplasma infection in the atypical hyperplasia was higher than that in the carcinoma tissue; the mycoplasma infection was positive in the gastric adenocarcinoma or in the papilloma, but was negative in the gastric mucoid carcinoma.

Table 1 Mycoplasma infection in different grades of gastric carcinoma

Grades of differentiation	Total number of cases	Infection of mycoplasma				
		Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
I-II	23	3	12	8	20	87
II-III	18	7	9	2	11	61
III	49	30	14	5	17	39
Total	90	40	35	14	50	56

In other gastric diseases, the total mycoplasma infection ratio was 31.5% (45/142). This ratio was significantly lower than that observed in the cancer tissue ($\chi^2 = 12.06$, $P<0.05$, Table 2).

Table 2 Comparison of mycoplasma infection in gastric carcinoma and in the other gastric diseases

	Infection of mycoplasma			
	Negative cases	Positive cases	Total number of cases	Ratio of positive (%)
Chronic superficial gastritis	34	13	47	28
Intestinal metaplasia	31	18	49	37
Gastric ulcer	32	14	46	30
Gastric carcinoma	40	50	56	56
Total number of cases	137	95	50(mean)	40(mean)

Mycoplasma infection in colon carcinoma and adenomarous polyp tissues

In the 58 cases of colon carcinoma, the total infection ratio was 55%. As in the gastric carcinoma described above, the colon carcinoma tissues with high differentiation had a higher mycoplasma infection ratio than that of low differentiation colon carcinoma tissues (Table 3, $P<0.05$).

In the 49 cases of adenomarous polyp, there were 10 cases with mycoplasma infection. The positive ratio was 20.4%. The difference between the infection ratio of colon carcinoma and that of adenomarous polyp was significant ($\chi^2 = 13.46$, $P<0.005$).

Table 3 Mycoplasma infection in different grades of colon carcinoma

	Infection of mycoplasma					
Grades of differentiation	Total number of cases	Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
I-II	42	15	15	12	27	64
II-III	8	5	2	1	3	37
III	8	6	2	0	2	30
Total	58	26	19	13	32	55 (mean)

Mycoplasma infection in other carcinoma tissues

Beside the gastriointestinal carcinomas, other cancer tissues from human esophagus, lung, breast and brain were also analyzed (Table 4).

Table 4 Mycoplasma infection in other carcinoma tissues

	Infection of mycoplasma					
Types of carcinoma	Total number of cases	Negative cases (-)	Positive cases (+)	Positive cases (++)	Total positive cases	Ratio of positive (%)
Esophagus	53	26	21	6	27	50.9
Lung	59	28	23	8	31	52.6
Breast	63	38	17	8	25	39.7
Glioma	91	53	27	11	38	41.0
Total	266	145	88	33	121	45.5

Some immunoperoxidase stainings of different carcinoma are shown in Figure 1. The low differential gastric cancer (ring cell cancer) was negative reacted with PD4 (A), but the gastric adenocarcinoma was positive (B).

Detection of mycoplasma DNA in positive specimen by PCR

We selected 3 positive and 3 negative specimens detected by immunochemistry for mycoplasma DNA amplification. The specimen DNA was extracted and PCR was carried out by using mycoplasma 16srDNA primers. The mycoplasma hyorhinis DNA was used as control. The analysis of agar gel electrophoresis showed that mycoplasma DNA was amplified from all three positive specimens, as well as from the positive control of mycoplasma DNA, but there was no mycoplasma DNA amplified from negative tissues, which should be 142bp in size (Figure 2). This result corresponded with that of immunohistochemical detection.

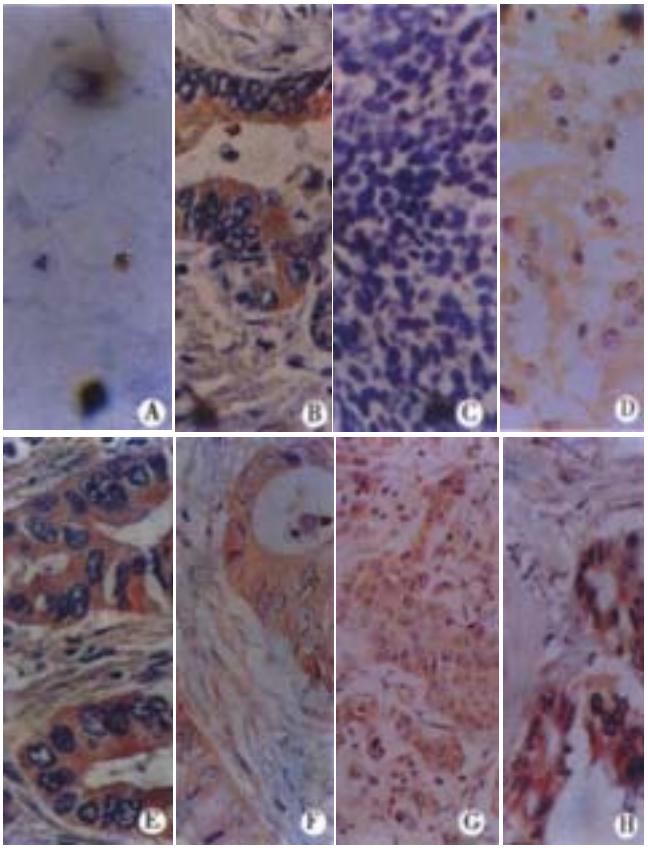

Figure 1 Immunoperoxidase stainings of different carcinoma tissues reacted with monoclonal antibody PD4 (× 400). Both A and B were gastric carcinomas. A (singnet-ring cell carcinoma) was negative, B (adenocarcinoma) was positive. C (glioma) indicated the negative reaction, D (glioma), E (lung cancer), F (esophagus cancer), G (breast cancer) and H (colon cancer) presented the positive reactions with antibody PD4.

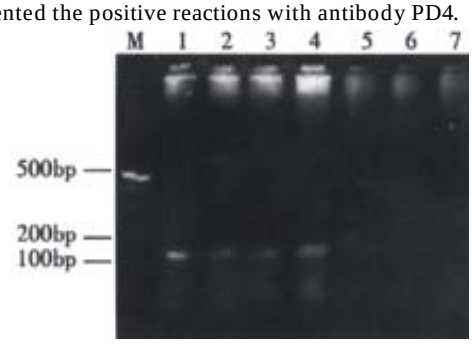

Figure 2 Amplification of mycoplasma 16srDNA from paraffin -N- embedded specimens. M: 100bp DNA Marker; Lane 1: Positive control; Lane 2-4: positive specimen; Lane 5-7: negative specimen. The arrow indicates the amplified mycoplasma DNA.

DISCUSSION

Mycoplasma exists widely in nature. Mycoplasma contamination in cultured cells is common and a major problem in bio-laboratory work. McAb, prepared with cultured tumor cells as immunogen and using these cells as selection targets, has been identified to be antibody against mycoplasma. Unfortunately, the molecules recognized by these antibodies are difficult to characterize and are usually researched as tumor-associated antigen. Meanwhile, these antibodies are regarded as tumor-specific[23,25]. McAb PD4 has been used as a tumor-specific antibody until its antigen was identified by protein sequencing. Because the characterization of tumor antigen is usually difficult, and mycoplasma contamination is common in cultured cells, researchers should be very careful in preparing antibody against tumor and take necessary steps to exclude the possibility of antibody binding to mycoplasma.

We observed that mycoplasma was present in 55% cases of gastrointestinal carcinomas ($P<0.05$) and 45.5% in otherdetected carcinomas. These results are similar to those presented by Philip[26] and Sasaki[27], where the rate of mycoplasma infection was 59.3% in ovarian cancer cases and 48% in gastric cancer, respectively. In gastrointestinal carcinomas, we found that cancer tissues with high differentiation had a higher mycoplasma infection ratio than that of low N differentiation cancers. The reason for this is unknown.

It may be logical to consider the correlation between the cancer and mycoplasma because of the high infection in the tumor tissues, but the role of mycoplasma as a causative or facilitative agent during tumor development has yet to be determined. Although the AIDS related *Mycoplasma fermentans* and *Mycoplasma penetrates* have been reported to induce cell transformation[15], and *Mycoplasma hyorhinis* was observed to increase invasiveness and inhibit cell contact inhibition *in vitro*[24], but direct evidence for a cause-and-effect relationship has not been discovered. In our recent work, we found that gastric cancer cell line MGC803 contaminated with *Mycoplasma hyorhinis* has a much higher capacity to form colonies on soft agar than that of MGC803 without mycoplasma contamination. Interestingly, we have observed McAb PD4 to inhibit colony formation of MGC803 with mycoplasma contamination, but no effect was observed on mycoplasma free MGC803 (unpublished). This result indicates that the protein recognized by McAb PD4 may play a critical role in colony formation increased by mycoplasma. Elucidating the mechanism by which PD4 inhibits colony formation will provide important information for understanding the relationship between mycoplasma infection and oncogenesis.

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