Mycoplasmapenetrans Were Isolated from Blood and Tumor Tissue of Cancer Patients

Liping Zhou, Xiushu Wen, Yeying Zhu, Jinwei Wang, Huangle Liu, Saifang Wang, Jing Wang

The First Affiliated Hospital of Wenzhou Medical College, Zhejiang, Wenzhou 325000, China

Abstract **Objective** To investigate the presence of *M. penetrans* in different crowd (tumors and non-tumor). **Methods** Blood and tumor tissue samples obtained from 171 patients with cancer were cultured and isolated for Mycoplasma species (*M. fermentans*, *M. pneumoniae* and *M. penetrans* and *M. pirum*). The cultured mycoplasmas were further examined according to their colony morphology, nPCR and DNA sequencing were also performed. A total of 115 non-tumor (71 cases non-tumor tissue and 44 cases blood samples from healthy subjects) were collected and analyzed for comparison. The positive samples were observed with electron microscopy. **Results** A typical *M. penetrans* strain isolated from blood of patient with gastric cancer was subjected to nucleic acid sequence analysis indicated *M. penetrans* strain with HF-2 strain no difference, the flask-shaped particles of *M. penetrans* were identified by electron microscopy. In the blood and tumor tissues the presence of *M. penetrans* were indicated by electron microscopy. In 171 patients, *M. penetrans* in both blood and tumor tissues were detected in 28 cases. Simultaneously, in 29 cases, *M. penetrans* were identified only in tumor tissues, and in 28 cases *M. penetrans* were only found in the blood. Thus the detection rates of *M. penetrans* were 33.3% (57/171) and 32.7% (56/171) respectively from tumor tissues and blood. In the 171 subjects, *M. penetrans* were detected in 85 strains (49.7 %, 85/171) only. In gastric carcinoma and colon cancer, the detection rate of *M. penetrans* was 59.2%(42/71),and 40.0%(18/45) in transitional cell carcinoma of bladder, in cervical cancer group it was 45.5% (25/55), compared with the control groups they had significance in statisticus. **Conclusion** The patients with cancers had high percentage of *M. penetrans* infections and their role needs to be explored further.

Key words *M. penetrans*; Isolated and Cultured; Tumor; PCR; Electron microscopy

Mycoplasmas are prokaryotes without cell walls of the class Molecutes. They are small, free-living, self-replicating organisms[1, 2]. Although mycoplasmas are found commonly in the oral cavity and as symbiotic gut flora, some species can cause acute and chronic illnesses when they penetrate into the vascular system and systemically colonize organs and tissues. For example, mycoplasmas, such as *M. penetrans*, *M. fermentans* and *M. pirum*, can enter a variety of tissues and cells and cause systemic signs and symptoms. Mycoplasmas have also been shown to have a complex relationship with the immune system [1]. They are very effective at evading host immune responses, and synergism with other infectious agents [2, 3]. A unique mycoplasma, termed mycoplasma penetrans was first isolated from the urine of a human immunodeficiency virus (HIV)-positive homosexual male patient[3, 4]. Recently, DNA from Mycoplasma hyorhinis(Mhr), *M. penetrans* and *M. fementans* has also been detected in tumor tissues[4]. Tsais *et al.* [5] and Zhang *et al.* [6] reported that infection of mycoplasma penetrans and *M. fementans* resulted in non-malignant morphological changes in the mouse embryonic cell line C3H after 6 weeks, with malignant alterations occurring in the eleventh week. At this point, the transformation became irreversible and a tumor formed in the eighteenth week, which demonstrated the ontogenetic potential of *M. penetrans* and *M. fementans* [6-8].

In the present study, we to investigate the presence different crowd (tumors and non-tumor)detection rate of *M. fermentans*, *M. pneumoniae* and *M. penetrans*.

Correspondence to: Liping Zhou
Email: zlpzlp19491949@163. com
Tel: 0577-88837538

We found the presence of *M. penetrans* infection in many patients suffering from bladder transitional cell carcinomas, gastric carcinoma, colon cancer, and cervical cancer than in non−tumor tissue.

MATERIALS AND METHODS

Patients and specimens Blood and tumor tissue samples were obtained from a total of 171 patients (HIV−negative, 95 female, 76 male) with tumors. Subjects in the study included 53 patients with gastric cancer, 18 patients with colon carcinoma, 45 transitional cell carcinoma of the bladder and 55 patients with cervical cancer. The mean age of all patients was 54±15 years. Subjects were investigated for mycoplasmas infections in their blood leucocytes with isolation and culture, and positive cases were further tested for a forensic polymerase chain reaction (PCR) procedure. Patients were investigated for the presence of Mycoplasma spp. tested for infections with the following species: *M. fermentans*, *M. pneumoniae* and *M. penetrans*. Subjects were identified through the department of Surgery, Urology of the Wenzhou Medical College, Zhejiang province. With comparison, a total of 115 non−tumor samples were obtained to isolate and culture of *M. penetrans*, which including blood samples from 44 healthy subjects, 20 patients with gastric ulcer, and 10 patients with procto polypus and 37 cases of cervical intraepithelial neoplasia (CIN) and cases of bladder polyp. The examined cases of a particular disease was selected according to the results of hematoxylin−eosin (HE) staining evaluated microscopically. The isolation and culture of *M. hyorhinitis* and *M. pirum* were not carried out. Blood was collected in citrate−containing tubes and immediately brought to ice bath temperature as described previously [9]. Samples were shipped refrigerated or on wet ice by over night courier for analysis. Whole blood (5ml) was used for preparation using isolation and culture of Mycoplasma and PCR (Wuxi Clone heredity research institute, china) as follows. Blood at room temperature for 30 min and after centrifugation at ×13000g for 2 min. The samples were used immediately stored at −20°C until use PCR technique and electron microscopy were used to detect positive blood and tumor tissue samples of patients with tumors.

Isolation and culture of Mycoplasma

All the blood specimens collected from patients were infused into PPLO broth (Difco, Detroit, Mich.) supplemented with 20% heat−inactivated horse serum and 10% yeast extract and cultured. After incubation in CO_2 at 37℃ for 4~5days, the medium turned yellow to distinguish positively, the negatively specimen to observe 30 days, respectively.They were distinguished from other mycoplasma strains. After a preliminary identification, the candidate strains were preserved at −20℃ for further study. The tumor tissue samples were filtrate infused into PPLO broth. And the same treatment with the specimens was come from control patients. Multiple mycoplasma tests were performed on all patients, 115 non−tumor samples were obtained to isolate and culture of *M. penetrans*.

Polymerase chain reaction of *M. fermentans* and *M. penetrans*

A polymerase chain reaction (PCR) technique was used to detect positive cultures samples of patients with tumors and control group. Amplification of target sequences was performed in a final volume of 25μl and each reaction mixture contained 10mM Tris−HCL (pH 8.8), 50mM KCL, 1.5mM $MgCl_2$, 0.1% Triton 0.1% Triton X−100, 200μM each of dATP, dTTP, dGTP, dCTP, 100pmol of each primer, and 0.5~1ng of DNA. Purified mycoplasma DNA (0.5~1ng of DNA) acted as a positive control for amplification. The amplification was carried out For 35 cycles with denaturing at 93℃ for 30s, annealing was performed at 55℃ for 30s, and extension temperature was 72℃ for 60s for the final extension. Negative and positive controls were used in each experimental run. The PCR product was analyzed by 1.2% agarose gel electrophoresis stained with 0.2μg/μl of ethidium bromide. The gel was immersed in 90 mM Tris−borate, and 2mM EDTA was subjected to 100V per 30 min. Subsequently, the gel was observed with a transilluminator to visualize the amplified products, and purified with an Agarose Gel DNA Purification Kit TaKaR (Wuxi, China).

DNA Sequencing

The sequence of the PCR positive product was confirmed identical to the published sequence by DNA sequencing (Shengon High Technology Corporation, Shanghai, china).

Electron microscopy observed

The pure culture of *M.penetrans* HF−2 strain from blood specimen of patients with gastric cancer were detected by transmission electron microscopy (TEM).The patients whose blood and gastric cancer tissue specimen with that were to confirmed positive of results of *M. penetrans*. These specimens were fixed with 2.5% glutaralglutaral and were processed with ultrathin section and then observed under H600 electron microscope. Also, the pure culture of *M. penetrans* from the blood was embedded and sliced, then observed by an electron microscope.

Statistical analyses

The results of samples was assessed by the chi−square test, and were considered as significant difference when $P<0.05$.

RESULTS

Isolation of Mycoplasma

Obtained specimens were cultured in liquid medium and identified to mycoplasma.Colonies of cells with a " fried egg" morphology were obtained (Fig.1).

Polymerase chain reaction (nPCR) verification and sequencing of the 16s rRNA gene of *M. penetrans* strains in clinical samples

Positive cultures from blood and tumor tissue samples were verified by PCR to contain *M. penetrans* (Fig.2). A typical *M. penetrans* strain isolated from blood of patient with gastric cancer was subjected to nucleic acid sequence analysis. Our results confirmed that the *M. penetrans* PCR product isolated from the blood of patients with tumors. In the current study, was identical to the nucleic acid sequence at positions 1−370bp from previously −isolated strain. A multi −sequence alignment was subsequently performed to identify the sequence identity of the 16s rRNA genes.

The result of the positive culture material from blood and tumor tissue of tumor patients. Lane1, Markers; Lane2, the positive control; Lane3~Lane5, the pos-

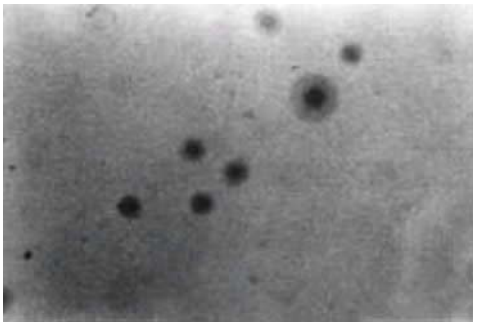

Fig. 1 "Fried−egg colonies", on the solid medium under the microscope (×40). Fried−egg colonies were identified from the blood sample of the patient with gastric cancer. The blood and tumor tissues sample was first cultured in the liquid medium for 4~5 days and then plated onto the solid medium and then incubated for another period of 72h and the Fried−egg colonies were identified.

Fig. 2 The results of Polymerase chain reaction (PCR) detection of *M. penetrans* in clinical samples and analyzed by electrophoresis in 2% agarose gel.

Fig. 3 Sequence of 16s rRNA gene of the *M.penetrans*. DNA sequence of MPe−nPCR products were come from the culture material of the blood of the case with gastric carcinoma. The sequence had 100% identity with that of HF−2 in NCBI database.

itive culture material from blood and tumor tissue of patients with gastric cancer and colon cancer ,respectively; Lane6 ~ Lane7, the positive culture material from tumor tissue of patient with cervical cancer; Lane8 ~ Lane9, the positive culture material from blood and tumor tissue of patients with bladder transitional cell carcinoma. on the 407bp witch lane control positive control showing no difference. Lane10, the negative control.

DNA Sequencing result

The 16s rRNA gene from *M. penetrans* was identical to the corresponding DNA fragment of *M. penetrans*. A typical *M. penetran* strain isolated from a patient with gastric cancer was subjected to nucleic acid sequence analysis. Our results confirmed that the *M.*

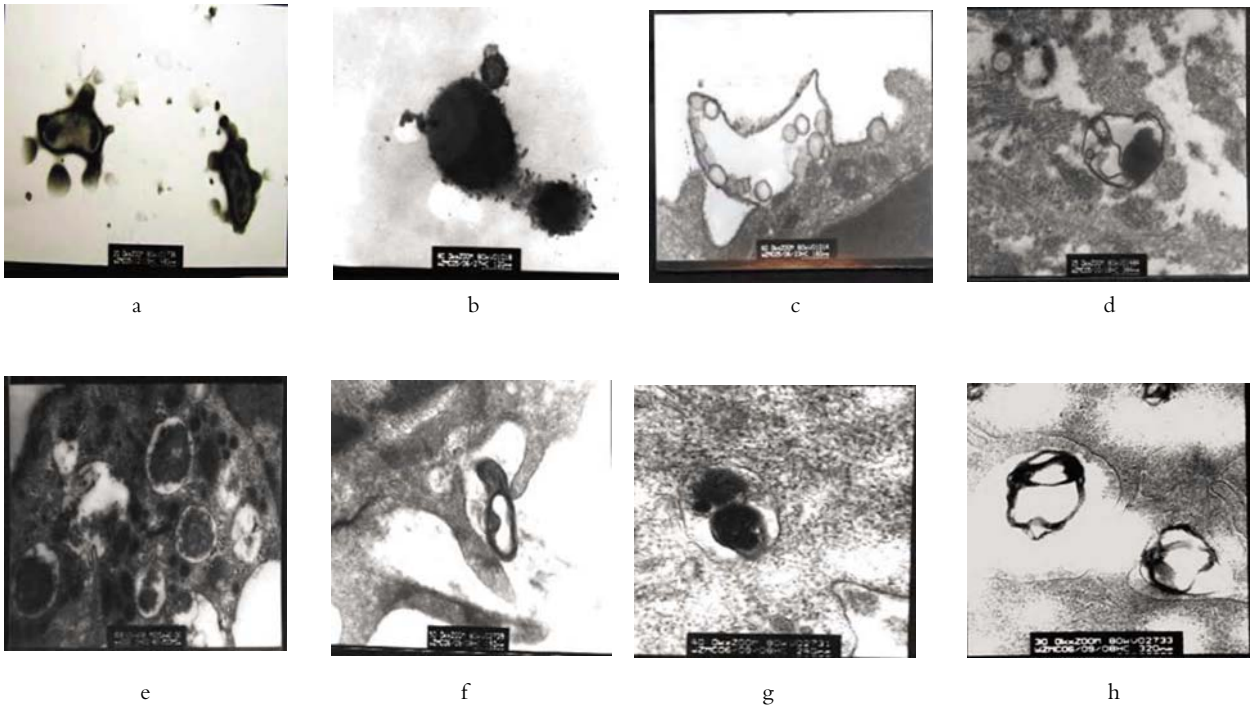

Fig. 4 a~g Electron microscopy micrograph of *M.penetrans*.

(a) Negatively staining electron micrograph of purified *M. penetrans* isolated from blood of patients with gastric cancer, showing characteristic morphologic elements were identified isolated from blood of patients with gastric cancer (Original transmission electron microscopic magnification, ×20000).

(b) The flask−shaped morphology of *M. penetrans*. The particles were divided into two parts (The bar below represents 300nm. Original transmission electron microscopic magnification, ×80000).

(c) A *M. penetrans* is indicated in the cytoplasm. A single triple−layered unit membrane is covered with capsular−like outer layer material, the globularity. It was found that mass proliferation of *M. penetrans* in phagocytic cell of patients with gastric cancer. (Original transmission electron microscopic magnification, ×60000).

(d) Showing *M. penetrans* penetrate to tumor tissue with gastric cancer. Note tumor tissue occupying dissolve state (Original transmission electron microscopic magnification, ×15000).

(e) The positive of M. penetrans cases in blood phagocytic cell of patients with colon cancer, with massinflammatory cell cytoplasm. (original transmission electron microscopic magnification, ×25000).

(f) Showing *M. penetrans*. penetrate to phagocytic cell from blood of patients with cervical cancer. Original transmission electron microscopic magnification, ×25000).

(g) A *M. penetrans* is indicated in the tumor tissue with cervical cancer. (original transmission electron microscopic magnification,×25000).

(h) A *M. penetrans* is indicated in the tumor tissue with bladder transitional cell carcinoma. (original transmission electron microscopic magnification,×25000). Note tumor tissue with gastric cancer, bladder transitional cell carcinoma and cervical cancer occupying dissolve state.

penetran PCR product isolated from the blood of patients with tumors was identical to the nucleic acid sequence at positions 1~370bp from previously isolated strain. A multi-sequence alignment was subsequently performed to identify the sequence identity of the 16s rRNA genes. The 16s rRNA gene from *M. penetran* was identical to the corresponding DNA fragment of M. penetran. The HF-2 gene in the NCBI data base, in the gene of standard strain with a sequential of HF-2 strain (Fig.3) showing no difference [9].

The detection of *M. penetrans* strains in clinical samples

The infection rate of *M. penetrans* in tissues of patients with tumor was up to 32.7% (57/171), but only was 3.1%(2/64)in control group.The difference was significant in statisticus (χ^2=22.1, P<0.001). In the 71 gastric carcinoma and colon cancer, the detection rate of *M. penetrans* was 59.2%(42/71) in blood or tumor tissues, but only was 3.3% (1/30) in 30 cases control group, the difference between the two groups was statistical significance (χ^2=27.8, P<0.01). The detection rate of *M. penetrans* in transitional cell carcinoma of bladder group was 40.0%(18/45), which had great significantly different from procto polypus (P<0.01). The detection rate of *M. penetrans* in cervical cancer group was 45.5% (23/55), which was significantly different from the CIN group (24.3%)(P<0.05). The infection rate of *M. penetrans* in blood of patients with tumor was up to 32.7% (56/171), but only was 4.2%(3/71)in control group. the difference was statistical significance (P<0.001). The *M. hyorhinis*, *M. fermentans* and *M. pneumoniae* Ureaplasma urealyticum, *M. hominis* were not detected. The result detection of *M. penetrans* in tumor tissues and blood of tumor patients and control group were presented in Table 1 and Table 2.

Electronic microscopy observation

The *M. penetrans* positive specimens of blood and tumor tissue from carcinoma patients were further processed with transmission electron microscopy (TEM) in order to detect *M. penetrans* particles (Fig.4). Spherical granules with a triple-layered membrane to detect *M. penetrans* particles (Fig.4). Spherical covered with were observed (Fig.4 a and b) and the flask-shaped particles of *M. penetrans* were identified (Fig.4c). In the flask-shaped particles of *M. penetrans* were identified with electron microscopy

DISCUSSION

Mycoplasmas are the smallest self-replicating polytrophic bacteria that lack cell walls. Wall-less bacteria were first described 100 years ago, and now over 190 species, widely distributed among humans, animals, insects and plants. As well known the cell walls of mycoplasma species absent, which further subdivide-into various strains [1, 2]. Of which least 8 had been found to cause directly or significantly influence many chronic diseases. These strains include Mycoplasma pneumonia, penetrans, pirum, genitalium, fermentans, hominis *M. hyorhinis*, and ureaplasma urealyticum. Mycoplasmas are although some species invadehost tissues and cells then act intracellular [10~12]. These microorganisms can produce a variety of effects on host cells and tissues [13, 14]. In addition to some species invade host tissues and cells and act intracellular [14, 15]. These microorganisms can produce a variety of effects on host cells and tissues. In addition to effecting cell growth and morphology, mycoplasmas are able to alter cellular metabolic, immunological and biochemical functions resulting in synergistic infection [14]. Some mycoplasma species, such as *M.hyorhinis* and *M. penetrans* produce a phospholipase whose continuous expression may be related with tumor occurrence [16~19]. Previous studies have shown that macrophages treated with interferon release pro-inflammatory cytokines affecting cell growth and morphology, mycoplasmas are able to alter cellular metabolic, immunological and biochemical functions resulting in synergistic infection [20, 21]. In addition, macrophages treated with interferon may release tumor necrosis factor alpha (TNF-α), and produce nitric oxide [22, 23]. TNF-α production is associated with stimulation by whole mycoplasmas and TNF-α induced apoptotic and anti-apoptotic pathways in endothelial cells, which have been relatively well studied [24~26]. However, their role in cancer was more difficult to establish.

In the 1990s two independent studies reported that

infection of mycoplasmas induced chromosomal alterations [1, 27]. Mycoplasmal infections apparently affected the fidelity of genomic transmission in cell division as well as checkpoints coordinating the progression of cell cycle events [1]. Using a murine embryonic (C3H) cell system, demonstrated that chronic infection by mycoplasmas induced chromosomal instability as well as malignant transformation of mammalian cells [6]. This mycoplasma-mediated oncogenic process had a long latency and demonstrated distinct multistage progression [28, 29]. Over expression of H-ras and c-myc oncogenes were found to be closely associated with both the initial reversible and the subsequent irreversible states of the mycoplasma-mediated transformation in C3H cells [30, 31]. Feng *et al.*[20] have developed a new paradigm for neoplastic processes based in vitro studies, and hypothesize that chronic infection or colonization by certain mycoplasmas may gradually induce malignant transformation and promote tumorous growth of mammalian cells [1,32~34]. Multiple cycles were necessary, probably because of the intracellular locations of mycoplasmas, as *M. fermentans* and *M. penetrans* [7]. Their inherent insensitivity to antibiotics and the slow-growing nature of microorganisms [13]. After recovery, these patients were no longer positive for mycoplasmal blood infections [9,10,13,].

Whether *M. penetrans* has ontogenic potential requires further investigation. Notably, the presence of mycoplasma, including M.hyorhinis, has been reported in several cases of human cancers [30]. Since little is known about the possible involvement of mycoplasmas in the pathogenesis of chronic diseases, it remains uncertain whether our findings implicated mycoplasma as causal agents,coinfactors or secondary infections in patients with carcinoma. However, it is clear that mycoplasma capable induce immune dysfunction[21]. Further studies must be undertaken to establish the relationship of mycoplasma with their host, and determine the possible synergism of these organisms with other biological or even chemical agents. *M. penetrans*, a rare bacterium that prior to present study had only been isolated from HIV-infected individual was also found in the blood and throat of non-HIV infected patient with primary anti-phospholipid syndrome,but the etiology and pathogenesis are unknown [31]. Mycoplasma-mediated multistage oncogenesis exhibited here shares many characteristics found in the development of human cancer [32]. Lipid-associated membrane proteins (LAMPs) of Mycoplasma penetrans rapidly induced macrophages to produce proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), IL-1 and IL-6 [33, 34]. The results of Feng *et al.* also suggested that high-level expression of TNF-α in cells induced by *M. penetrans* lipid extract of PK-digested. LAMPs is associated with rapid activation of transcriptional factors NF-κB and activator protein 1(AP-1). LAMPs from *M. fermentans* and *M. hominis* showed stimulatory effect on the GR response to Dex in these cells. It is well-known to us that steroid hormones, such as estrogen and androgen, promote cell proliferation in sexual organs and play an important role in the induction of cancer formation in these tissues [1]. Once induced, chromosomal alterations continued to accumulate both in cultured cells and in animals without the continued presence of the transforming microbes. Mycoplasma-mediated multistage oncogenesis exhibited here shares many characteristics found in the development of human cancer. Although the effects of mycoplasma LAMPs on steroid receptor transactivities are not as strong as the effect of cAMP, the biological importance of the long-term effect on steroid receptorfunctions in human hosts with chronic mycoplasma infection or colonization could be significant [34].

Today these results strongly suggested *M. penetrans* was more common in bladder transitional cell carcinomas, gastric carcinoma, colon cancer, and cervical cancer than in non-tumor tissue. First of all, this study is to investigate the presence of M. penetrans in 171blood samples from patients with tumors with techniques, including isolated cultured, nPCR and DNA analyses. Then the tumor tissue and blood of patients with cancers were analysed by electron microscopy, we found *M. penetrans* in individual with gastric and genital tract carcinoma. Specially, *M. penetrans* was isolated from blood or tumor tissues. It is important that the isolation and culture mycoplasma from blood and tumor tissue can not only avoid false positive results, but also make possible further in order to prevent abuse of antibiotic susceptibility test [28, 29]. Therefore, a combination ap-

proach is preferable to the use of PCR alone in the detection of mycoplasma strains in blood and tumor tissue of carcinoma patients. If so, that needs to be stated more clearly. Such as, "Diagnosis of mycoplasma infection may also increase survival rate of patients post-surgery". This result demonstrating that *M. penetrans* not only isolated from blood but also from tumor tissue of carcinoma patients. Interestingly, this study indicated a typical *M. penetrans* strain isolated from blood of patient with gastric cancer was subjected to nucleic acid sequence analysis and the results indicated *M. penetrans*-HF-2 witch HF-2 strain no difference. And in the flask-shaped particles of *M. penetrans* were identified by electron microscopy. In the blood and tumor tissues, the presence of *M. penetrans* were indicated by electron microscopy. The results suggested that a high percentage of tumor patients have *M. penetrans* infections. *M. penetrans* nfections may be an important cofactor in the pathogenesis with tumor occurrence, and their role needs to be explored [1, 2]. Much remains unknown about the role of mycoplasmas in human disease [1, 2]. This has been demonstrated *M. penetrans* in blood and tumor tissue of carcinoma by electron microscopy micrograph. The demonstrated *M. penetrans* in blood and tumor tissue of carcinoma patients´ tissues, provides an area for novel investigation into the actions of mycoplasma in cancer.

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