

PCR Analysis of *Mycoplasma fermentans* and *M. penetrans* in Classic Kaposi's Sarcoma

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Sir,

Kaposi's sarcoma (KS) is a multicentric vascular neoplasia characterized by macules, nodules and angiomatoid plaques mainly present on the skin surface. Four KS variants are recognized: classic or Mediterranean; endemic or African; the variant following on renal transplant or immunosuppressive therapy; and, lastly, the epidemic or AIDS-associated variant. The variants differ in the sites involved, course of the disease and in their prognosis. Genetic, immunological, environmental and viral factors have proven to be relevant in the aetiology and pathogenesis of all KS variants. In addition, further possible interacting factors such as mycoplasma infections have come to attention for the single variants.

Mycoplasmas are prokaryotes belonging to the class *Mollicutes*. They are the smallest free-living self-replicating bacteria known (1). Mycoplasmas have no cell wall and a very limited genome of between 600 and 1500 kbp. The fact that they have such a limited genome makes them highly dependent upon their host for survival.

Recent studies have implicated certain mycoplasma species as possible co-factors in a number of clinical conditions. *Mycoplasma fermentans*, *M. hominis* and *M. penetrans* have been isolated from individuals suffering from a variety of diseases including chronic fatigue syndrome, rheumatoid arthritis and Gulf War syndrome (2, 3).

In 1989, Lo et al. isolated a bacterium in AIDS-associated patients with KS which they initially denominated 'mycoplasma-like' (4). Subsequent works have demonstrated that this bacterium, isolated from KS lesion biopsy material, belongs to a strain of the already well-known *M. fermentans* (5). Wang et al. found a higher seropositivity for *M. penetrans* in homosexuals with AIDS-associated KS as compared with homosexuals with AIDS but who had not developed KS (6).

Because of the high incidence of classic KS in northern Sardinia, we decided to investigate the presence of mycoplasmas in these patients, by searching for nucleotide sequences belonging to *M. penetrans* and *M. fermentans*. *M. penetrans* and *M. fermentans* were searched for using the polymerase chain reaction (PCR) in DNA samples from diseased and healthy skin biopsy tissue taken from patients with classic KS and a group of patients designated as controls.

MATERIALS AND METHODS

Clinical specimens

Our study comprised 29 patients with classic KS (19 men and 10 women, age range 59–81 years). Samples from each patient had undergone haematoxylin and eosin histological examination, which had confirmed diagnosis and had shown the grouping to be positive for HHV8 and negative for HIV. For the purposes of this study, one biopsy sample was taken from affected skin and one from healthy skin for each patient.

The same sampling protocol was effected on 42 individuals comprising an age- and sex-matched control group of patients suffering from different skin pathologies and not affected by sexually transmitted diseases. Amplification was performed with species-specific primers, and patients were investigated for the presence of *M. fermentans* and *M. penetrans*. This study was approved by the ethical committee.

DNA isolation

Skin tissues were treated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) (TE), 1% SDS and 200 $\mu\text{g ml}^{-1}$ proteinase K at 55°C for 3 h. Phenol-chloroform-isoamyl alcohol (25:24:1) was added to each sample to extract the DNA. The DNA was precipitated with a 1/10 volume of 3 M sodium acetate and 2 volumes of absolute ethanol and incubated at –20°C overnight. The samples were subjected to centrifugation at 16 000 rpm for 15 min and then washed in 70% ethanol.

The DNA pellets were resuspended in 100 μl of sterile TE. Concentration and purity were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm. DNA samples were then stored at –20°C until use.

PCR amplification

Each DNA sample was subjected to a total of three PCR amplifications. One used primers for gene regions coding for actin to establish the effective presence of skin DNA and lack of inhibition. Subsequently, two PCRs for each sample were set up using species-specific primers for *M. fermentans* and *M. penetrans* so as to discover the presence of nucleotide sequences of mycoplasmatic origin. The nucleotide sequences belonging to *M. fermentans* were searched for using a pair of primers that identify the 'Tuf' region of the gene while the nucleotide region of *M. penetrans* was searched for using primers that identify the 'P35' region of the gene (7).

Amplification of target sequences was performed in a final volume of 25 μl and each reaction mixture contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 200 μM each of dATP, dTTP, dGTP, dCTP, 100 pmol of each primer, and 0.5–1 ng of DNA. Purified mycoplasma DNA (0.5–1 ng of DNA) was used as a positive control for amplification. The amplification was carried out for 35 cycles with denaturing at 94°C. Annealing was performed at 60°C (*M. penetrans*) or 55°C (*M. fermentans* and actin). Extension temperature was 72°C in all cases. Finally, product extension was allowed at 72°C for 10 min. Negative and positive controls were used in each experimental

run. A volume of 20 μ l from each reaction was separated on a 1% agarose gel stained with 0.2 μ g/ μ l of ethidium bromide. The gel was immersed in 90 mM Tris-borate, and 2 mM EDTA was subjected to 100 V per 30 min. Subsequently, the gel was observed with a UV transilluminator to visualize the amplified products.

RESULTS AND DISCUSSION

Skin biopsies were taken from healthy and affected skin of 29 patients with classic KS and of 42 patients suffering from other skin pathologies. All the biopsies were subjected to DNA extraction by phenol-chloroform-isoamyl alcohol and subsequently tested using PCR. They showed no traces of DNA sequences belonging to either *M. penetrans* or *M. fermentans*, either in the healthy or in the affected skin of either grouping.

The negativity of the data obtained for both patient groupings would lead to the conclusion that *M. penetrans* and *M. fermentans* are mycoplasmas which are not frequently present at skin level. Our findings would seem to exclude these mycoplasmas from a role as co-factors in the development of classic KS. It must be pointed out, however, that serological investigation would be needed as confirmation of this latter hypothesis, as our results do not exclude the possibility of the presence of these mycoplasmas in other organs and tissues.

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