Detection of the Jaagsiekte Sheep Retrovirus by RT-PCR in naturally ovine pulmonary adenocarcinoma-affected sheep and in apparently healthy in contact-sheep

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SUMMARY

The ovine pulmonary adenocarcinoma (OPA) is a naturally occurring contagious lung tumour probably caused by the Jaagsiekte Sheep Retrovirus (JSRV), which resembles to bronchiolo-alveolar carcinoma in humans and for which alone a histopathological diagnosis can be made when clinical signs are already apparent. Macroscopic and histological examinations as well as the JSRV detection by RT-PCR were performed in lungs sampled from 10 spontaneously OPA-affected Iranian crossbred sheep from herds of Fars province (which exhibited respiratory distress and coughing with discharge of copious mucous exudates from nostrils and also firm greyish nodules or masses in the lung lobes) and from 10 apparently unaffected animals in close contact with diseased animals in the same herds. Tumoral masses were evidenced in the cranio-ventral or in the caudal lobes from lungs of all diseased sheep and the presence of pulmonary adenocarcinoma was effectively histologically confirmed (cubical or columnar cells eventually forming adenomatous or papillary structures lining alveoli and bronchiolo epithelia). Furthermore, the JSRV detection in lungs by RT-PCR was positive in 100% of cases. On the other hand, no lesion was noticed in lungs from the 10 apparently healthy in contact-animals but positive PCR results were observed in 4 cases. These results confirm the role of the JSRV in the OPA development, but also suggest that the infection would occur before the installation of a discernable lung tumour.

Keywords: Ovine pulmonary adenocarcinoma, sheep, histopathology, RT-PCR, Jaagsiekte Sheep Retrovirus, oncogene.

Introduction

Ovine pulmonary adenocarcinoma (OPA) has striking analogies to some forms of human adenocarcinoma [9]. It is a worldwide contagious lung cancer that is caused by a β-retrovirus, the Jaagsiekte sheep retrovirus (JSRV) [10]. The mechanism by which the virus causes oncogenic transformation of the lung epithelial alveolar type II and the non-ciliated bronchiolar cells (Clara cells) is still not completely understood but may involve expression of oncogenic retroviral structural proteins in tumour cells or pro-viral insertional mutagenesis [9]. This virus is unique among retroviruses because of its specific tropism for the differentiated lung epithelial cells, and it is the only virus known to cause a naturally occurring lung adenocarcinoma. Moreover, the only sites where the JSRV is highly expressed in vivo are the transformed lung epithelial cells. In lymphoid cells, the JSRV can be detected in naturally infected sheep before the onset of clinical disease and even before the development of discernible neoplastic lesions [4]. A routine diagnosis is solely based on histological diagnosis that has been possible only when clinical signs become apparent and not during the preclinical period of the disease. This is due to a lack of JSRV-specific antibodies, as demonstrated by Western blotting [8, 16] and to the failure to detect JSRV proteins outside the lung tumour, as demonstrated by blocking ELISA and immunohistochemistry techniques [9]. The use of RT-PCR demonstrated the tissue JSRV dissemination in the lympho-reticular system and in blood mononuclear cells from terminally ill sheep during natural or experimental infection [7]. The aim
of the present study is to investigate the presence of the JSRV in lungs from affected sheep with obvious pathological evidence of natural OPA but also in the organs of in-contact animals with no pathological signs of pulmonary tumour.

Materials and Methods

ANIMALS AND TISSUE SAMPLING

Ten naturally affected sheep with OPA and 10 in-contact animals apparently healthy and without pathological evidence of pulmonary tumour were included in the present study. All sheep were Iranian crossbred from herds of Fars province, Iran, with mainly intensive breeding system. The diseased and apparently healthy animals were stemming from the same herds and were completely in contact together. Main clinical signs of affected animals were respiratory distress and coughing with discharge of copious mucopurulent exudates from nostrils. Intensive husbandry facilitates horizontal transmission by the copious nasal discharge.

The lungs were collected from slaughterhouse or during necropsy. A gross evaluation was performed and revealed the presence of firm greyish nodules or masses in the lung lobes from all the diseased animals. Appropriate tissue samples were immediately collected for histopathological and PCR studies. There was no delay between death and lung sampling because the sampling was done just after the slaughter of animals. For the histological analysis, tissue samples were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm in thickness, stained with haematoxylin and eosin (HE) and examined by light microscope using 40 X fields (high power fields). The other tissue samples were frozen at -70°C before PCR detection.

PCR ASSAY

RNA extraction

Total RNA from lung tissues (0.1 g) was extracted using RNA isolation kit (Cinnagen, Iran) [2]. Briefly, 100 mg of lung tissues were added to 1 mL of RNX Solution provided by the kit. After homogenizing, 200 μL chloroform were added and mixed by vortex and the aqueous phases were separated. The RNA samples were precipitated by equal volume of isopropanol and the aqueous phases were discarded. The RNA samples were washed twice with 75% ethanol. The isolated RNAs were eluted in double distilled water containing 1 mM EDTA and 0.1% DEPC (Diethyl pyrocarbonate), and then stored at -70°C. Each RNA sample was treated with 10 U of DNase I, RNase free (Fermentas), as suggested by the manufacturer, before the RT-PCR.

RT-PCR

Synthesis of cDNA was carried out in 20 μL reaction volumes as follows. Total RNA (5 μL) and primers P1 and P2 (20 pmol) that snapped a 229-bp region internal to the gag gene (position 1598 to 1826 of JSRV) [18] were incubated at 70°C for 5 min, then transferred on ice. The mixture was transferred to lyophilized RT-Accupower tube (Bioneer, South Korea) and 13 μL diethyl pyrocarbonate treated distilled water were added. After addition of reverse transcriptase (20 μg/μL) and incubation at 42°C for 1 hour, the enzyme was inactivated at 94°C for 5 min. Thereafter, a volume of 5 μL of cDNAs was used in PCR using PCR premix (Bioneer, South Korea) containing 1 U Taq DNA polymerase, 250 μM of each dNTP, 10 mM Tris- HCl pH 9, 40 mM KCl and 1.5 mM MgCl2 in 20 μL reaction. The program used in the thermal cycler (Biorad) associated an initiation step at 94°C for 5 min., 35 cycles of 94°C for 45 seconds, 57°C for 1 min. and a final extension of 72°C for 2 min. The plasmid pTZ57R/T containing a part of the JSRV gag gene (nucleotide 953 to 3029) was used as the positive control. Amplified products (7 μL) were detected by 2% agarose gel electrophoresis in TBE (Tris-borate-EDTA) buffer in the presence of 0.5 μg / mL of ethidium bromide.

Restriction enzyme digestion

According to the manufacturer’s instructions, the PCR-gag products (25 μL) were digested with 13U of ScaI (Fermentas) at 37°C for 2-4 hours and the digestion products were analyzed by 2% agarose gel electrophoresis and stained with ethidium bromide.

Results

During the gross examination, lesions of the lungs from sheep with OPA consisted in solitary or multiple, firm and dry nodules to greyish firm coalescing masses in the cranioventral or caudal lobes. The cut surface of the tumour masses was often moist with exuding frothy fluid (figure 1). By contrast, no macroscopic lesion was observed in lungs from apparently healthy sheep.

Histopathologically, the lesions were characterized by the formation of nodular lesions in which the alveoli and bronchioles were lined with cubical or columnar cells, often forming adenomatous or papillary structures (figure 2). Desmoplasia or fibroplasia of interstitial tissue was prominent in advanced lesions and in some sections nodular masses of myxoedematous tissue were intercalated between foci of desmoplasia. In addition, mild to moderate infiltration of mononuclear cells, especially of lymphocytes, was observed and caused the thickening of the interstitial tissue of the affected alveoli. Some large and swollen macrophages were present mostly in the apparently normal alveoli surrounding or in proximity to the neoplastic affected areas. Bronchoalveolar lymphoid tissues (BALTs) were diagnosed as hyperplastic and prominent in some sections (figure 3). In parallel, no tumour and no inflammatory reaction was evidenced in lungs from apparently healthy animals.

A 229 bp DNA fragment obtained by RT-PCR compatible with amplification of the JSRV gag gene using P1 and P2 specific primers was obtained from all the naturally OPA-affected sheep (figure 4). The characterized ScaI digestion profile of the gag gene fragment (production of 2 bands of
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131 bp and 98 bp, respectively) was reproduced from all RT-PCR positive samples (figure 4) and confirmed the JSRV detection. Moreover, the RT-PCR assay was also positive in 40% (4/10) of lung extracts prepared from apparently healthy sheep in contact with diseased animals.

Discussion

OPA is a naturally occurring bronchoalveolar adenocarcinoma of sheep and rarely in goats [3]. It cannot be controlled effectively because there are no diagnostic test in living animals and consequently no effective prophylactic tool [14]. The incidence of the disease is usually 2-5% [17], although it can reach 30% in some areas of the United Kingdom [9]. OPA is considered as an animal model for human lung cancer because of important similarities with some forms of lung adenocarcinomas: the understood of the molecular bases of OPA could provide a possible framework to explain some aspects of pulmonary carcinogenesis [9, 12]. In human, lung cancer is the leading cause of cancer death, accounting for the lives of more than a million people yearly world wide [5]. The majority of lung cancers are associated with cigarette smoking; however, about 10% of lung cancers in men and 20%-25% in women are not associated with smoking [16]. The unique epidemiological features of bronchoalveolar carcinoma suggest that the causes of this carcinoma are different from those of other lung malignancies. In addition to possible genetic and familial predispositions, the multiclonality of at least some multifocal cases of bronchoalveolar carcinoma might suggest an environmental carcinogen or an infectious agent [1]. Human papillomavirus DNA has been detected in approximately a third of bronchiolo-alveolar carcinoma cases [6]. Bronchiolo-alveolar carcinoma is now defined as a type of adenocarcinoma with a pure bronchiolo-alveolar carcinoma growth pattern and no evidence of stroma, vascular, or pleural invasion. The neoplastic cells grow as a single
layer along the walls of the terminal airways and alveoli [15].

In the present study, the JSRV was detected in all 10 lungs from OPA affected sheep and in 40% of lungs from in-contact sheep without gross or microscopic lesions. GONZALEZ et al. [4] described the detection of the JSRV proviral DNA in the blood and in lympho-reticular system tissues of clinically affected and unaffected sheep with pathological evidence of natural OPA and in-contact animals with no evidence of pulmonary neoplasia. In their study, the JSRV detection was positive in lung tumours and in lymphoid organs and the number of positive PCR results was greater in affected sheep than for in-contact animals. They concluded that the JSRV can be detected in naturally infected sheep before the onset of clinical disease and even before the development of discernible lung tumours. Our results are in agreement with these previous findings. It seems that the subclinical JSRV infection can reach a high prevalence in flocks affected by OPA.

The importance of retroviruses in understanding carcinogenesis is due to the fact that common mechanisms are involved in retrovirus-induced neoplasms and in tumours that lack retroviral aetiology. For example, many proto- oncogenes transduced by retroviruses (e.g., ras, myc, abl, erbb) are now known to be activated in certain human cancers [13]. The analysis of 249 human lung tumours (including 129 bronchio-alveolar carcinomas, 65 adenocarcinomas, and 41 squamous cell carcinomas) by immunohistochemistry using a rabbit antiseraum targeted the major capsid protein of the Jaagsiekte sheep retrovirus (JSRV) revealed immune-reactive cells in 22% of the cases: 30.2% (39/129) of bronchio-alveolar carcinomas and 26.2% (17/65) of adeno-car cinomas were positive whereas the JSRV was not detected in the other types of lung tumours (squamous cell carcinomas and others) as well as in not tumoral lesions and in normal lung tissues [9]. Although other virology and molecular studies are not available, the possible involvement of the JSRV or of a related retrovirus in some form of bronchio-alveolar carcinoma is attractive. It would be also possible that a protein which the expression is markedly increased in transformed cells cross-react with the JSRV antiseraum. Alternatively, a human endogenous retrovirus (HERV) immunologically related to the JSRV might be over expressed in the neoplastic cells [2].

Despite the different arguments strongly pointing the JSRV as in aetiological agent for OPA, it was unclear for many years whether this virus alone was sufficient to induce lung cancer. The lack of a suitable cell culture system to propagate the JSRV in vitro has hampered efforts to establish with certainty the aetiology of the tumour. Nevertheless, PALMARINI and FAN [9] obtained an infectious molecular clone of the integrated DNA form of JSRV (termed "JSRV21") from a spontaneous OPA case. The use of a derivative of pJSRV21, in which the cytomegalovirus immediate early promoter was used to drive high-level expression of viral proteins (pCMV2JS21) in transiently transfected human 293T cells, has allowed bypassing the absence of cell culture system. The intra-tracheal inoculation of viral particles produced by the transfected 293T cells lead to the development of clinical OPA in 2 of the 4 treated newborn lambs after 4 months [9]. Moreover, the presence of the viral proteins in the lung lesions were confirmed by immunohistochemistry and the molecular JSRV markers were found by PCR in the tumour lesions [11]. This evidence provided the first conclusive demonstration that the JSRV is necessary and sufficient to induce OPA, and it opened the way to exploring the mechanisms of oncogenesis.

In agreement with previous reports [4, 8], the JSRV was systematically evidenced in OPA neoplastic lungs by PCR. In addition, the JSVR was detected in lungs from apparently unaffected in-contact-animals with a relatively high prevalence (40%). Several hypotheses can be proposed. Firstly, these animals may be in a preclinical stage, the viral multiplication leading to neoplastic transformation. Secondly, some predisposing factors would be required such as a host immune relative deficiency allowing the multiplication of even poorly virulent JSRV strains. However, the precise mechanisms and factors that regulate the genesis and progression of the tumour related to the JSRV remain to be elucidated.

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