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Original contribution

Absence of markers of betaretrovirus infection in human pulmonary adenocarcinoma☆

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Summary A proportion of human pulmonary adenocarcinomas has been shown previously to express an antigen related to the Gag protein of a betaretrovirus, Jaagsiekte sheep retrovirus, that causes ovine pulmonary adenocarcinoma. To investigate further the hypothesis that a retrovirus might be present in human lung adenocarcinoma, we examined specimens from patients with lung cancer for evidence of retroviral infection by immunohistochemistry, reverse transcriptase–polymerase chain reaction, immunoblotting and cDNA library screening. Thirty-eight percent of the tumor samples analyzed were positive by immunohistochemistry for Gag-related antigen of Jaagsiekte sheep retrovirus. However, this antigen was not detected by immunoblotting using the same antiserum. In addition, plasma samples from the patients did not contain antibodies reacting with Gag proteins from Jaagsiekte sheep retrovirus or other betaretroviruses on immunoblots. Reverse transcriptase–polymerase chain reaction identified the expression of endogenous betaretroviruses in tumor tissue and in normal lung tissue, but no specific provirus was associated with tumor. Expression library screening did not identify the Gag-reactive antigen. This study has confirmed the expression of a Jaagsiekte sheep retrovirus Gag–related antigen in some human lung tumors but additional evidence of betaretroviral infection was not obtained. While these data do not rule out a role for a retrovirus in human pulmonary adenocarcinomas, they suggest that, if such a virus is present, it is unrelated to known betaretroviruses.

1. Introduction

The relative incidence of primary adenocarcinoma of the lung has increased over the last few decades, and it is now the commonest subtype of lung cancer in Japan and North America [1,2]. The reasons for this are unclear, and although a link with tobacco smoking is recognized, the association is weaker than for other forms of lung carcinoma [3], which may implicate additional environmental risk factors in its etiology.

Ovine pulmonary adenocarcinoma (OPA, also known as Jaagsiekte) is a contagious lung cancer of sheep caused by a betaretrovirus, Jaagsiekte sheep retrovirus (JSRV) [4,5]. OPA arises from type II alveolar pneumocytes and bronchiolar Clara cells and is characterized by increased surfactant production in the lung, which may be discharged from the nostrils of affected sheep when the animal's head is lowered [5]. The lung fluid contains a high concentration of

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infectious JSRV and transmission of OPA is thought to occur predominantly via the respiratory route. Oncogenesis in OPA is induced by the JSRV envelope glycoproteins, which, in addition to their role in cellular entry, transform epithelial cells in vitro [6,7] and in vivo [8,9].

OPA has a number of histologic similarities with some forms of human lung adenocarcinoma and this has led to speculation that a retrovirus related to JSRV might be etiologically linked with the human disease [4,5,10,11]. Particular similarities have been noted with mixed adenocarcinomas comprising papillary, acinar, and bronchioloalveolar features [11]. In older reports, human lung tumors with this pattern were defined as bronchioloalveolar carcinoma (BAC). In 2000, the World Health Organization's definition of BAC changed to refer specifically to adenocarcinomas that exhibit only bronchioloalveolar growth and are strictly non-invasive. This change in definition should be recognized when comparing older reports with more recent studies. True BAC is very rare and has been proposed to represent a pre-invasive step in the development of peripheral lung adenocarcinomas [12,13]. Under the current definition there are therefore important differences between OPA and BAC since OPA is clearly an invasive process and metastasis to intra-thoracic lymph nodes is common. Therefore, OPA is perhaps best classified as an adenocarcinoma of mixed type with a BAC component.

A number of previous reports have described evidence linking a retrovirus related to JSRV with human lung adenocarcinoma. Immunohistochemical studies using antibodies raised to the capsid proteins of JSRV and other betaretroviruses gave positive cytoplasmic labeling in around 30% to 40% of lung adenocarcinoma cases [14,15]. These included cases described as BAC as well as other pulmonary adenocarcinomas. In contrast, specimens from other tumors and nonneoplastic lung diseases were rarely positive by the same analysis. These data suggest that an antigen related to JSRV Gag, possibly retroviral in origin, is present in human pulmonary adenocarcinoma.

Other reports have described polymerase chain reaction (PCR) and reverse transcriptase (RT)–PCR analysis of human lung tumor specimens using primers specific for JSRV sequences. Initial studies using this approach failed to detect the virus [16,17]. However, JSRV-related sequences were subsequently reported in whole blood DNA from blood donors and from individuals infected with HIV-1 [18]. A small panel of BAC and small cell lung cancer specimens were also tested but were found to be negative for JSRV sequences. A further study [19] has reported JSRV sequences in human tissues including BAC tumor, normal lung and normal liver. The authors suggested that geographical origin may be important in determining the presence or absence of JSRV. These recent reports of positive detection require further confirmation since JSRV is not recognized as a zoonotic agent, despite the possibility of repeated exposure of sheep farmers and abattoir workers to high concentrations of the virus present in lung fluid and affected lungs.

In order to investigate the hypothesis that a betaretrovirus related to JSRV is etiologically linked to human pulmonary adenocarcinoma, we examined a panel of patients with lung tumors for evidence of betaretroviral infection using a variety of techniques. Tumors were assessed for the expression of proteins related to JSRV Gag by immunohistochemistry (IHC) and immunoblotting, and for expression of betaretrovirus RNA by RT-PCR with degenerate PCR primers. The presence of betaretrovirus-reactive antibodies in the patients' plasma was investigated by immunoblotting. In addition, we attempted to clone the Gag-reactive antigen by screening cDNA expression libraries prepared from positively labeling tumors.

2. Materials and methods

2.1. Patients and tissues

The project was carried out with appropriate local ethical and managerial approval. Informed consent was obtained from 28 patients attending the Thoracic Surgical Unit at the Royal Infirmary of Edinburgh for surgical resection of pulmonary carcinomas. The resection specimens were sampled by placing small portions of fresh tumor and adjacent normal lung tissue in RNAlater solution (Ambion, Warrington, UK). Specimens were then fixed by inflation with neutral buffered formalin and processed routinely for classification and staging. Subsequently, further sections were taken from the tissue blocks containing the primary tumor for IHC. Blood samples (10 mL) were collected from each patient and from 6 healthy volunteers into EDTA tubes and plasma samples were stored at −20°C.

2.2. Immunohistochemistry

Immunohistochemical analysis of tumor sections was performed on 26 of the 28 tumor specimens using a rabbit antiserum against the CA (Gag) protein of JSRV [15]. Labeling was performed with the preimmune and postimmune sera (diluted 1:300), incubated on slides at 4°C overnight and developed using a standard methodology using biotinylated goat anti-rabbit IgG and diaminobenzidine visualization. The pathologist evaluating the sections was not blinded to which of the antisera was used (ie, preimmune or postimmune).

2.3. Immunoblotting

Protein extracts from lung tissue were prepared using the PARIS protein isolation method (Ambion) and quantified by BCA assay (Pierce, Cramlington, UK). JSRV preparations were produced by transfection of human 293T cells with plasmid pCMV2JS$_{21}$ and harvesting the culture supernatant 48 hours later as previously described [20]. The 293T cell line was cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich, Poole, UK) supplemented with 4 mM