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Short Communication

Detection of Mouse Cytomegalovirus in Adenocarcinoma Bearing Razi/A Mice: Molecular and Pathological Studies

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ABSTRACT

Despite a lot of research, the etiology and progression of breast cancer remain incompletely understood. Recently, human cytomegalovirus (HCMV) was reported as a risk factor for breast cancer. The aim of this study was to know whether breast cancer could be caused by cytomegalovirus or not? In this experiment seventeen samples of RAZI/A mice with spontaneous breast cancer were being gathered from laboratory animals department. Histopathology and polymerase chain reaction (PCR) tests were done on breast tissue samples. Formalin-fixed tissue specimens were obtained from mouse normal breast tissues (n:17) and mouse mammary tumors (n:17). Detection of mouse cytomegalovirus was done by the pUC57-MCK-2 plasmid. Our histopathology data showed Adenocarcinoma type B in mouse with mammary tumors. There was a significant difference between mice with spontaneous breast cancer and control by Pearson Chi-Square (Value: 17.000[b] and P=0.000). More research will be needed to determine the effect of cytomegalovirus on breast cancer.

Keywords: Mouse Cytomegalovirus, Breast Cancer, Human cytomegalovirus, MCK-2 Gene

INTRODUCTION

Infectious agents, mainly viruses, are among the few known causes of cancer and contribute to a variety of malignancies in worldwide (Pagano *et al* 2004). A human retroviral analogue of MMTV and Epstein-Barr virus (EBV) has been reported to occur in up to 38 and 50% of human breast cancers, respectively (Mant *et al* 2004). Similarly, it would be of interest to know whether breast cancer could be caused by cytomegalovirus or not? More researches indicate that human cytomegalovirus (HCMV) infection can modulate signaling pathways associated with oncogenesis (Asanuma *et al* 1996, Hamprecht *et al* 1998). HCMV proteins and nucleic acids have been detected in several malignancies, including breast, prostate, colon, mucoepidermoid carcinoma of salivary

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glands and pleomorphic rhabdomyosarcomas in trp53+/2 mice (Soroceanu *et al* 2011, Melnick *et al* 2012, Price *et al* 2012). Dr. Ann Richardson showed that women with breast cancer had higher cytomegalovirus antibody levels than women without breast cancer (Richardson *et al* 1997, Richardson *et al* 2004, Cox *et al* 2010). Recently So-derberg-Naucler and her colleagues worked on 73 human breast cancer samples to investigate whether human cytomegalovirus (HCMV) infection is associated with several malignancies or not? Their experiments confirmed observations by Harkins et al, that demonstrating high HCMV protein expression in breast cancer. They found that 100% of primary breast cancer samples were HCMV positive in most neoplastic cells in sentinel lymph node metastases of breast cancer. They suggested that HCMV protein expression is maintained in most metastatic cells and further evaluation need to understand possible mechanisms of virus contributing to breast cancer tumorigenesis and metastatic disease (Harkins *et al* 2010, Taher *et al* 2013). Murine cytomegalovirus (MCMV) is a dsDNA virus with ~230 kb genomic size (Saederup & Mocarski 2002). Data showed that approximately 50% of genes identified in mouse cytomegalovirus were homologous with human cytomegalovirus (Brocchieri *et al* 2005, Rawlinson *et al* 1996, Ho *et al* 1991, Alford *et al* 1993). Cytomegalovirus has a tropism for the salivary gland (Ho *et al* 1991, Sweet 1999, Saederup *et al* 2002). RAZI/A mice is part inbred mice (IR-Rsi (2C)) that among different strains of mice are more susceptible to breast cancer (Festing 1987). We hypothesized that MCMV infection might be associated with breast tumor in mice. In this study, the expression of MCK-2 genes of mouse cytomegalovirus has been evaluated in RAZI/A mouse breast tissue samples.

MATERIALS AND METHODS

AccuPrep® Genomic DNA Extraction Kit (BIONEER, Corea), GF-1 Plasmid DNA. Extraction Kit (Vivantis, Malaysia). Accuprep Genomic DNA Extraction Kit (Bioneer, Corea) cat: 1201. Mineral-oil (Sigma M8410). *E. coli* GM2163 Fermentas. DNA Ladder 100-3000, Fermentase. Ampicillin (Sina Clon, Iran).

Animal and tissue procedures. Animal usage was approved by the Animal Care and Use Committee of Razi Vaccine and Serum Research Institute. Female RAZI/A mice between 5 to 8 weeks old were kept under standard procedure. Tissue samples were prepared from normal and mouse mammary tumors. All Samples were kept both in formalin-fixed paraffin-embedded and also at -80 °C for molecular works (Figures 1-A, 1-B).

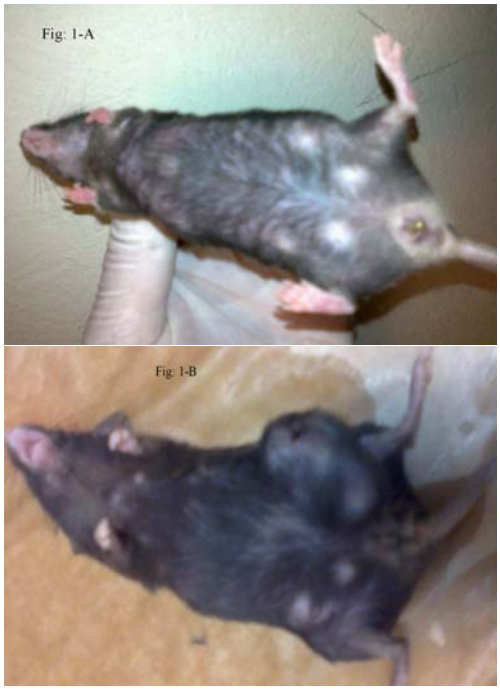

Figure 1. RAZI/A mouse. 1-A, Normal RAZI/A mouse; 1-B, Grossly mammary tumors in RAZI/A mouse (Red arrow).

Tissue preparation. Tumor biopsies were fixed with formalin, dehydrated in a series of 70–100% ethanol, cleared in xylene and embedded in paraffin. Sections of 4 μm were processed for hematoxylin and eosin (H&E) staining (Luna *et al* 1968).

Histological Classification. All of seventeen spontaneous mouse mammary gland tumors were histological classified according to the Thelma Dunn classification (Sass & Dunn 1997).

DNA Extraction from Mouse Breast Tissues. Collected samples (25~50 mg) from mouse breast tissues disrupted with a sterile surgical blade (BB 510) on sterile aluminum foil (put on ice), and were placed in a clean 1.5 ml tube. DNA extraction was performed by genomic DNA extraction kit (Bioneer, Corea) as the manufacturer's protocol. DNA was stored -20 °C until use.

Construction of pUC57 plasmid containing MCK-2 gene as Positive control. pUC57 is a common used plasmid cloning vector in E. coli and contained the ampicillin resistant gene. The vector length is 2,710 bp and is isolated from E. coli strain DH5α. The sequence of MCK-2 gene (862 bp) available in the Genbank databases (accession numbers: EMBL: AM236130 and AM236132) were synthesized and cloned in pUC57 vector by Vivantis company (Kuala Lumpur, Malaysia). The pUC57-MCK-2 plasmid was used as positive control.

Preparation of E. coli DH5 α Competent Cells and cell transformation. E. coli DH5α competent cells were prepared by using cacl2 method and transformation was performed by Sambrook protocol (Sambrook & Russell 2001).

pUC57-MCK-2 Plasmid Extraction from *E. Coli*. Plasmid extraction performed by GF-1 plasmid DNA extraction kit (Vivantis, Malaysia) as the manufacturer's protocol. DNA was stored at -20°C until use.

PCR Protocol. Primers targeting K-2 gene of murine cytomegalovirus were designed from conserved regions. Forward primer: CAT GAT GTA CGT GGC CGA TG. Reverse primer: TAC TGT ATC CAC ACC GTG GG. Amplicon: 180 bp (from 360 to 540). The PCR was done as follows: initial denaturation at 95 °C (3 min) followed by 35 cycles with a denaturation at 95 °C (20 s), annealing temperatures at 57 °C (1 min) and an elongation step at 72 °C (1 min). The program ended with 7min at 72 °C for primer elongation. A 25 µl overlay of sterile mineral-oil was added to the mixtures. All PCRs were carried out in an Eppendorf Mastercycler Gradient (Hamburg, Germany).

RESULTS AND DISCUSSION

Histopathological Findings. To evaluate of the mammary gland was done by hematoxylin and eosin (H&E) staining. To determine the effect of mouse cytomegalovirus on mammary gland H&E staining was done on both mammary gland of normal mice (n:17) and from mice with spontaneous mammary gland tumor (n:17). Mammary gland tumor samples were multinodular and easily separated from surrounding tissue. Tumor tissues showed adenocarcinoma type B. In general, most slides showed papillary ingrowths, irregular epithelial structure, cyst formation and small glandular. The lesion composed of solid sheets of epithelium with little or no glandular differentiation. Infiltration of mononuclear cells were prominent within the lobular mass (Figures 2-A, 2-B).

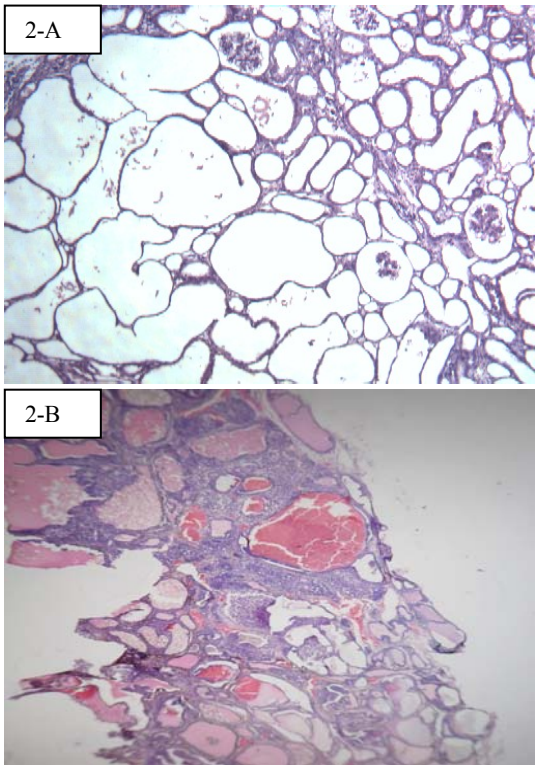

Figure 2. Histology of mammary gland: 2-A, Normal mammary gland, mouse H&E, ×40 objective) 2-B, Mammary gland adenocarcinoma, mouse, solid appearance of the proliferating cells. (arrow), (H&E, ×400 objective).

Polymerase Chain Reaction (PCR) Results. Seventeen samples from mouse with normal breast

tissues and seventeen samples from mouse with mammary tumors were evaluated by PCR. The PUC57-MCK-2 plasmid was used as positive control in this experiment. Data showed the prevalence of mouse cytomegalovirus gene sequences (MCK-2) of control and tumors was 16.5% (3 of 17) and 88.2% (15 of 17), respectively (Figures 3: 3-A and 3-B). There was a significant difference between mice with spontaneous breast cancer and control by Pearson Chi-Square (Value: 17.000[b]; P=0.000).

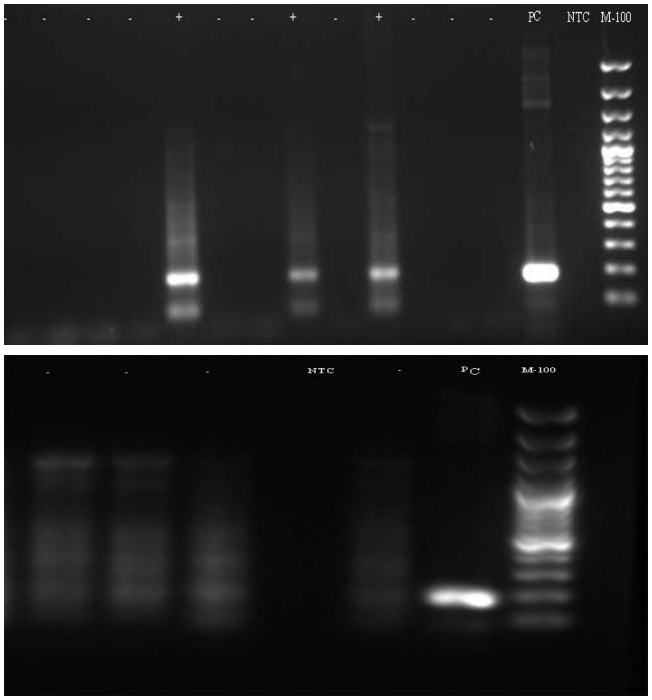

Figure 3-A. The Result of PCR from Mammary gland Tissues, PCR result from 17samples of normal mouse Mammary gland tissues (pUC57-MCK-2 Plasmid was used as a positive control). Data show 16.5% infection with MCMV. Abbreviation for this figure: M 100 (ladder started from 100 bp (1500bp)), NTC (non-template control, or negative control), pC (Control plasmid or positive control), + (positive sample), - (Negative sample).

Researchers have shown there is variation in the incidence of mammary tumors among different strains of laboratory mice. The low incidence of mammary tumors has been demonstrated in the BALB/c strain, whereas over to 100% of C3H females develop mammary tumors when they reach nine months of age (Dean *et al* 2007). Spontaneous mouse mammary tumors were originally classified under a scheme developed by Thelma Dunn. Letter designations (A, B, C, AB, L, P, Y, etc.) were used for classification. Types A, B and C were the most common types of spontaneous mammary tumor in mice (Sass and Dunn, 1979). Lualhati and colleagues evaluated the effect of human CMV on breast cancer. Surgical biopsy specimens were obtained from 38 normal breast individuals and 39 breast carcinoma patients. They have detected human CMV expression specifically in glandular epithelium (63%) of normal adult breast cases but in the neoplastic epithelium (97%) patients with ductal carcinoma in situ (DCIS) and infiltrating ductal carcinoma (IDC) cases evaluated (p = 0.0009) (Harkins *et al* 2010). Research by Booth and colleagues on the prevalence of antibody to murine cytomegalovirus (MCMV) in wild mice (n: 468) from diverse regions of Australia showed 90% of MCMV infection (Booth et al. 1993). Further study in the laboratory mice has found infection of mice with 2 strains of cytomegalovirus (Gorman *et al* 2006). In this experiment, for the first time the effect of MCMV was evaluated in mice with spontaneous breast cancer. All the samples were being gathered from RAZI/A mice during three years and samples were being kept at -80 °C and also were fixed in formalin. Based on of Smith and his colleague's research, PUC57-MCK-2 plasmid was chosen for positive control of PCR in this study instead of mouse CMV. The MCK-2 gene is pro-inflammatory chemokine-like protein. Smith and colleagues analyzed the level of sequence variation in selected genes of 26 isolates of MCMV. They reported that MCK-2 is gene with low-levels of variation in mouse CMV (Fleming *et al* 1999, Saederup & Mocarski 2002, Smith *et al* 2006). In this study histopathology data showed adenocarcinoma type B in all seventeen samples from mice with spontaneous mammary tumor. Most of the slides showed papillary ingrowths, cyst formation and small glandular. The lesion included well differentiated papillary growth within a dilated duct and infiltration of mononuclear cells were prominent within the lobular mass (Figures 2-A, 2-B). Molecular work by PCR shows murine CMV expression was higher in mouse with mammary

tumors than control tissues and raising the possibility that mouse cytomegalovirus infection may be involved in the neoplastic process. In this study the prevalence of mouse cytomegalovirus was higher in mouse breast tissue samples with spontaneous breast cancer 88.2% (15 of 17) than in normal breast tissue samples (3 of 17). There was a significant difference between control and mice with spontaneous breast cancer (P>0.05). We conclude that Cytomegalovirus might be causing breast cancer in mice and more research is needed to understand possibilities of virus contributing to breast cancer.

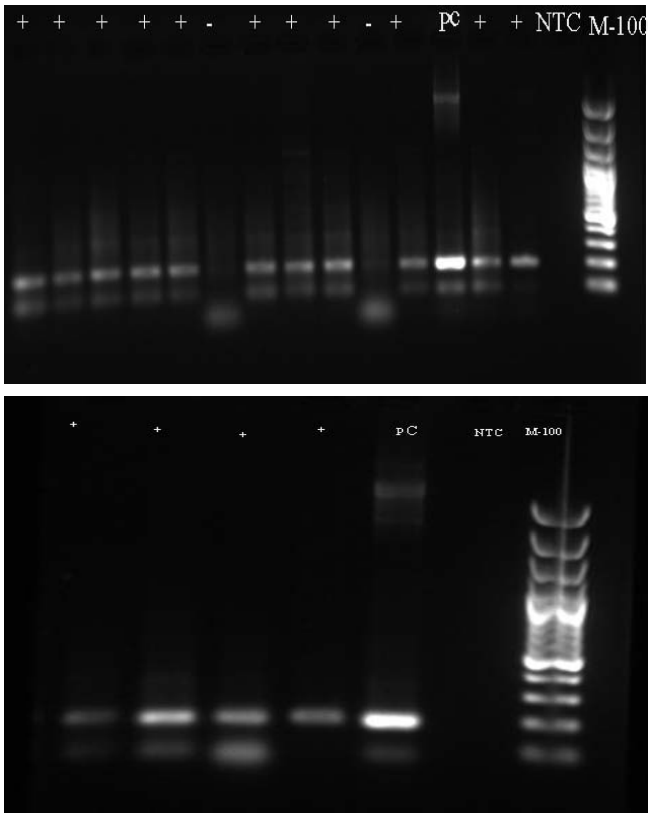

Figure 3-B. **The Result of PCR from Mammary gland Tissues,** PCR result of seventeen samples of mouse mammary tumors (pUC57-MCK-2 Plasmid was used as positive control). Data show 88.2% infection with MCMV. Abbreviation for this figure: M 100 (ladder started from 100 bp (1500bp)), NTC (non-template control, or negative control), pC (Control plasmid or positive control), + (positive sample), - (Negative sample).

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