**Short Communication**

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

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Received 30 Sep 2012; accepted 06 Jan 2013

**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 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 \textit{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 \textit{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 \textit{et al} 1996, Hamprecht \textit{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
Cytomegalovirus has a tropism for the salivary gland
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).

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 commonly 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 DH5α 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; P=0.000).

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 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, Saedergup & 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).

Acknowledgment

This work was sponsored by Razi Vaccine and Serum Research Institute (the project number 2-18-18-90004).

Special Thanks go to Dr. Ezzi, for his precious help and comments during the writing manuscript from Razi Vaccine & Serum research Institute.

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