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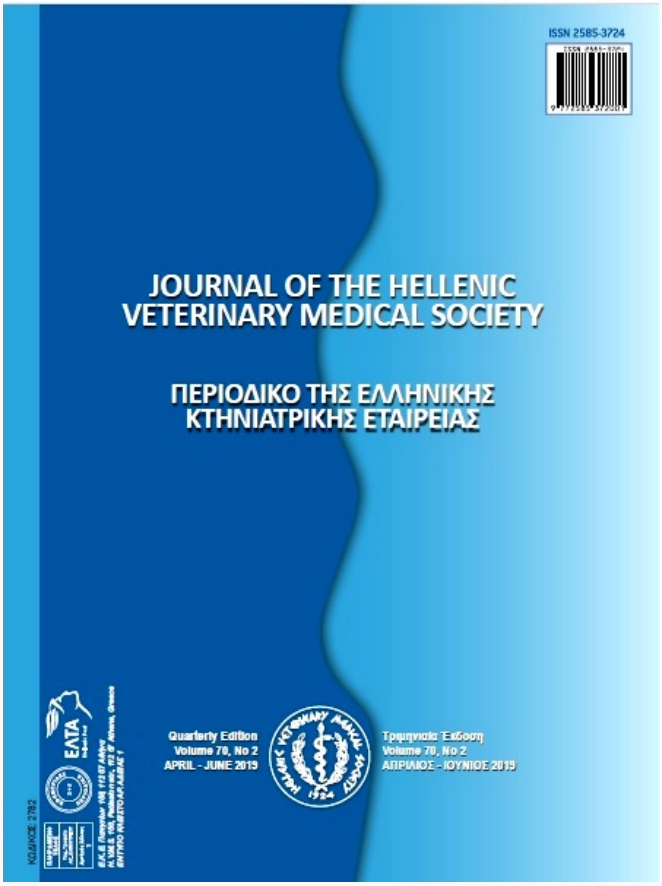

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Research article
Ερευνητικό άρθρο

The survivin gene expression in neoplastic hepatocytes from chickens infected with Marek's virus

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ABSTRACT. P53 protein is one of the main proteins in apoptosis pathway. The role of survivin protein in inhibition of P53 in different human cancers has been proved. The expression of survivin can be a main marker in diagnosis and prognosis of different human cancer. Until now, the survivin gene expression in birds infected with Marek's disease was not investigated. In this study, liver tissue samples of chickens infected with Marek's disease virus (MDV) was collected. The identification of MDV was carried out with PCR and histopathologic examination. After identification of MDV, the pathogenicity of infected virus was investigated with specific primers targeted on 132 bp tandem repeat. After this, the survivin gene expression was examined in neoplastic liver samples by Real-Time PCR. The PCR amplification of tumor liver samples showed that all samples were infected with MDV. The result of histopathology and amplification of 132 bp tandem repeat in tumor samples showed that the chickens were infected to pathogenic MDV. Results showed that the survivin gene expression in neoplastic hepatocytes was significantly higher than normal hepatocytes. In conclusion, survivin gene expression can be utilized as a suitable biomarker in diagnosis of Marek's disease in birds. It seems that viral *meq* oncogene in Marek's disease can play role in induction of survivin expression in this disease that it is necessary to be proved.

Keywords: Chicken, Gene expression, Marek's disease, Survivin.

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INTRODUCTION

Marek's disease (MD) is a lymphoproliferative disease in chickens that caused by oncogenic herpesvirus (Tulman et al., 2000). This virus replicates in the lymphoid and epithelial tissues (Biggs, 2001). The virus can form lymphoma in different organs and caused anemia and immunosuppression in chickens, especially layers and breeders (Calnek, 2001). The economic importance of this infection is related to mortality, decreased egg production and hatchability in chickens (Schat and Nair, 2013).

The disease firstly described by Joseph Marek, a veterinarian, in a cockerel with nervous sign (Marek, 1907). The virus can induce different and variety forms. The acute classic or visceral, neurological, cutaneous and ocular are common forms of this infection. The prevalent and main form of MD is acute classical form that is characterized by lymphoma in different visceral organs like liver, heart, lungs, spleen, kidney, bursa of Fabricious, proventiculus and gonads (Calnek, 2001).

Survivin, a newly characterized member of the inhibitor of apoptosis proteins (IAP), is a bifunctional protein which regulates cell proliferation and suppresses apoptosis (Altieri and Marchiso, 1999). Survivin is highly expressed during embryonic development and fetal tissues (Altieri, 2015). There is limited information about survivin expression in most normal differentiated tissues (Ambrosini *et al*., 1997). Survivin is also overexpressed in a wide variety of human neoplasms, suggesting that reactivation of the survivin gene frequently occurs in neoplasms (Ambrosini *et al*., 1997).

Currently, survivin protein expression is being used as a prognostic factor in several human neoplasms (Johson and Howerth, 2004). High survivin expression in human neoplasms associated with more aggressive behavior, reduced response to chemotherapeutic agents and decreased survival times, compared with neoplasms that are survivin negative (Altieri, 2015). Manipulation of survivin regulation and expression may leads to the development of new immunotherapy and gene therapy strategies for the treatment of neoplasms (Altieri and Marchiso, 1999). Until today, there is no information regarding survivin gene expression in chicken.

The purpose of this study is to demonstrate the survivin gene expression in chicken's neoplastic tissues and the comparison of the expression rate of survivin in normal and neoplastic tissues.

MATERIALS AND METHODS

After identification of chicken flock with MD, the suspected chickens were transferred to laboratory. The chickens euthanized and necropsied for sampling. The chickens that showed nodular lesions in visceral organs were selected for further examination. For each flock, 3 liver samples with nodular lesions were taken. The sampling was carried out for the detection of MDV and identification of pathogenicity by PCR test, pathological examination and survivin expression by Real-Time PCR. The samples were collected from 5 suspected flocks. Furthermore, liver samples from 5 apparently healthy chickens were collected as negative control.

DNA was extracted from nodular and normal liver tissues using a commercial DNA extraction kit (AccuPrep Genomic DNA Extraction Kit**,** Bioneer co., South Korea).

PCR was carried out to amplify fragments of 314 and 434 bp of the antigen A (specific for MDV-serotype 1) and 132 bp tandem repeat of MDV, respectively. The sequences of the antigen A and 132 bp tandem repeat primers were as following: forward primer: 5′-GAG GTA CCT CAT GGA CGT TCC ACA-3′; reverse primer: 5′-ACA TTC TTT TCG TTG GCG TGG TAT-3′ (Antigen A) (Bechker *et al*., 1992). Forward primer: 5′-TAC TTC CTA TAT ATA GAT TGA GAC GT-3′; reverse primer: 5′-GAG ATC CTC GTA AGG TGT AAT ATA-3′ (132 bp tandem repeat) (Bechker *et al*., 1992). PCR amplification was performed in PCR buffer containing 2 m*M* MgCl2, 200 μ*M* each dNTPs, 1 μ*M* each primer, and 1.0 unit of *Taq* polymerase (Fermentas, Germany) in a 25 μL total reaction volume. The 100 bp DNA ladder plus (Fermentas, St. Leon-Rot, Germany) was used. The amplification was carried out in a thermal cycler (Mastercycler Gradient, Eppendorf-Nethel-Hinz GmbH, Hamburg, Germany) using the following conditions: an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation, annealing, and extension at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec respectively, and a final extension at 72°C for 5 min. The PCR product was then analyzed by electrophoresis in 1% agarose gel and visualized under UV light after staining with ethidium bromide.

In this study live HVT vaccine (FC126 strain belonged to serotype 3, Merial, France) and sterile water

were utilized as positive and negative, respectively.

For analysis of lymphocytic infiltration in liver, the tissue samples were fixed in neutral 10% formaline solution, embedded in paraffin and cut into 4 micrometer thick sections. After deparaffinization, the sections were stained with hematoxylin-eosin (H & E) method (Bancrof and Stevens, 1996).

Total RNA was extracted from normal and neoplastic hepatocytes using a RNeasy kit (Qiagen, Valencia, CA, USA) and cDNA was synthesized using a QantiTect Reverse Transcription Kit (Qiagen, Valencia,CA, USA) according to manufacturer's instruction.

Real-time RT-PCR for Survivin gene was performed using specific QuantiTect primer assay (Fowl survivin, Cat no. QT00595896, Qiagen, Valencia, CA, USA) using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA).

The GAPDH was used as housekeeping gene. For the amplification of the GAPDH gene a pair of primers was synthesized by Bioneer (South Korea). The primers' sequences for chicken GAPDH were: Forward primer, 5'-GGTGGTGCTAAGCGTGTTA-3'; reverse primer, 5'-CCCTCCACAATGCCAA-3', resulting in an amplified product of 179 bp (Accession No. X01578) (Li *et al*., 2005).

Quantitative Real-Time RT-PCR was performed in a 20 μL reaction volume containing 10 μL of the SYBR Green PCR master mix (Fermentase, Germany), 1.5 μL of the RT reaction mixture, 0.5 μL each primers and 7.5 μL dH2O using the Applied Biosystems StepOnePlus™ Real-time PCR system (Foster City, CA, USA).

Amplification program included of initial denaturation step at 95°C for 10 minute, flowed by 40 amplification cycles consisting of denaturation at 95°C for 15 s, annealing at 60°C for 60 s, and extension at 72°C for 30 s. Real-Time PCR assays were carried out in triplicates and repeated three times. Relative gene expression was calculated using the standard curve method.

All data were analyzed by the statistical package for social sciences (SPSS) version 18.0 software (Chicago, Inc, USA) using one-way analysis of variance (ANOVA) statistical method at $p< 0.05$.

RESULTS

In suspected chickens to MD, a variety of macroscopic lesions was observed in visceral organs where focal nodular and diffuse whitish spots on the liver were featured (Figure 1). In apparently healthy chickens no macroscopic lesions were observed in liver or other organs.

Figure 1. The gross pathological lesions on liver in suspected chicken to MD.

The microscopic section of liver represents focal neoplastic infiltration which it is accompanied with pleomorphic cells. The neoplastic foci are consisting of lymphocyte and lymphoblast infiltration with different size, from small to medium (Figure 2). In apparently healthy chickens with normal liver tissues no microscopic lesions were seen.

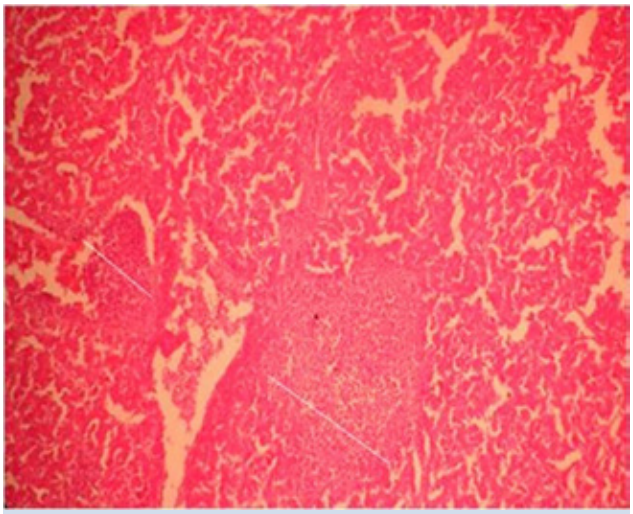

Figure 2. The neoplastic foci in liver of MDV infected chickens (H&E).

The 314 bp fragment related to antigen A of MDV was amplified in all liver samples with neoplastic lesions similar to positive control. In positive control, the 132 bp tandem repeat was not amplified while in all clinical samples the 132 bp tandem repeat with band size of 434 bp was amplified. The 314 and 434 bp fragments were not amplified in samples from apparently healthy chickens (Figure 3).

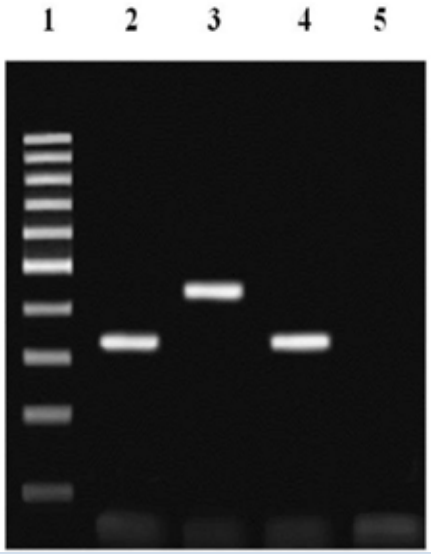

Figure 3. The PCR amplification of the Antigen A and 132bp tandem repeat of MDV in tissue samples from chickens (Lane 1: DNA ladder marker (100 bp) ; lanes 2: positive samples for Antigen A of MDV; Lane 3: positive samples for 132 bp tandem repeat; Lane 4: positive control (HVT Vaccine); and Lane 5: negative control).

The results showed that survivin is expressed in normal and neoplastic hepatocytes in chickens. The expression rate of survivin in normal and neoplastic hepatocytes was 0.113±0.136 and 1.440±0.500, respectively. The comparison of expression rate showed that there is a significant difference in survivin gene expression in normal and neoplastic hepatocytes in apparently healthy and MDV infected chickens (Figure 4).

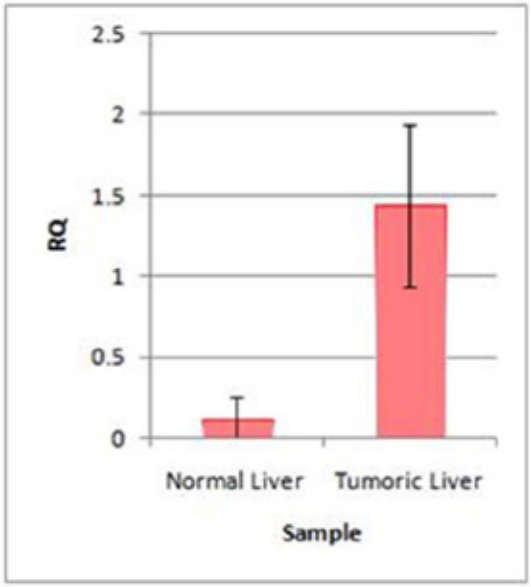

Figure 4. The expression of survivin gene in normal and neoplastic hepatocytes in chicken.

DISCUSSION

Marek's disease is the most important infectious disease in layers and characterized by development of lymphomas in different visceral, epithelial and nervous organs (Schat and Nair, 2013). PCR is the rapid and sensitive test to detect the presence of MDV in clinical samples (Shahzad, *et al*., 2007). Becker *et al* (1992) had designed one pair of primers that it could distinguished the pathogenic and non pathogenic MDV by a PCR assay amplified a 343 bp fragment of 132 bp tandem repeat. Zhu *et al* (1992), Becker *et al* (1992) and Kozdrun *et al* (2001) have declared that the PCR tests can be used for the identification of virulent MDV DNA in pathological samples and to distinguish between the virulent and non-virulent MDV in chickens. Therefore, in this study were utilized two pairs of primers designed on secretory antigen A and on the tandem repeat of 132 bp in order to identify MDV DNA in clinical samples and to differentiate the detected DNA, respectively. PCR and pathology results estimated that the sampling stage has been in stage of tumorigenesis of virus which in this time point, the viral antigen has been detected in tissues with microscopic lesions. Although this study increased knowledge regarding the Marek's disease of birds, it can be also used as a model about human cancer.

P53 protein is one of the most fundamental proteins for apoptosis induction and tumor inhibition (Shaikh and Niranjan, 2015). In normal condition, activation of apoptosis pathway and cell proliferation preserves normal size and performance of tissues (Nachmias *et al*., 2004). Mutation in protein P53 could lead to inactivation of apoptosis pathway, disorder of cell biochemical reactions and abnormal growth of cells (Shaikh and Niranjan, 2015). Survivin has been identified as a member of IAPs family (Johnson and Howerth, 2004). In this study, survivin gene expression in MDV infected hepatocytes in chickens was investigated and evaluated by sensitive and precise method of Real-Time PCR. Survivin plays role in cell proliferation, decrease of apoptosis of tumor cells, resistance to chemotherapy, radiotherapy and recurrence of many human cancers (Alteiri, 2015; Johnson and Howerth, 2004; Lei *et al*., 2010). But until today, it hasn't been investigated in tumor cells of birds. This gene is available in most germinal cells or in differential cells and most of cancers (Altieri, 2015). High expression of survivin in cancer cells indicates its prominent role for apoptosis inhibition during development of tumor. The gene is considerably expressed almost

in all human tumors (Verdecia *et al*., 2000). The cancers that have been reported high expression of this gene include lung, breast, gastric, esophagous, thyroid, colon (Altieri, 2015), larynx, pancreatic (Kato *et al*., 2001), bladder (SWANA et al., 1999), uterine (Saitoh *et al*., 1999), ovarian (Yoshida *et al*., 2001), liver (Ito *et al*., 2000), non-melanoma skin (Grossman *et al*., 1999) cancers. Tamm *et al* (2000) investigated expression of survivin in 60 lines of human cancer cells and observed high expression of survivin for all 60 samples. Furthermore, it has been recently indicated that anti-survivin drugs sensitizes tumor to chemotherapy by induction of apoptosis (Olie *et al*., 2000). Thus, survivin is studied as a suitable antigen for both diagnosis and treatment.

In current study, survivin gene was expressed and compared in liver normal and tumor cells but its expression rate was significantly higher in tumor cells. It seems the high expression of survivin in liver tumor cells in MDV infected chickens could be attributed to the interaction of *meq* oncogenes of Marek's virus with P53 protein. Recently, correlation between *meq* protein of MDV and HSP70 has been proved as an inductor of neoplasm (Zhao et al., 2009) and CDK2 and Rb proteins have been proved as regulators of cell cycle (Altieri, 2015; Shaikh and Niranjan, 2015, Nachmais *et al*., 2004; Zhao *et al*., 2009). Also, it has been shown that *meq* protein could shorten G1 phase of cell cycle (Nair and Kung, 2004). In this regard, some studies indicate that viruses' oncogenes are effective in high expression of survivin. So, already relationship between protein X of hepatitis B virus in human hepatocarcinoma (Marusawa *et al*., 2003), protein Tax type 1 of T-cell leukemia virus (Kawakami *et al*., 2005) and protein E6 of popilloma virus (type 16) in uterine cancer (Borbely *et al*., 2006) with the overexpression of survivin gene has been indicated. Also, it has been shown that oncogene protein LMP2A of Epstein-bar virus can induce the expression of survivin and the prohibition of apoptosis (Hino *et al*., 2008). Therefore, the high survivin expression in liver tumor cells could be due to the activation by *meq* oncogene of MDV.

In this study, has been shown that survivin expression in liver tumor cells has significantly been higher than liver normal cells. Survivin expression in normal cells can be explained by the presence of very small numbers of stem cells (Altieri 2005) in liver tissue that could be used for the preservation of tissue homeostasis. Liver tissue is always regenerating cells to preserve the tissue homeostasis against different chemical materials including drugs and bacterial toxins. Expression of survivin gene in this organ could be associated with the existence of premature and undifferentiated cells at primary step of cell regeneration. In addition, an inhibitor of apoptotic proteins has been observed in some cells of soft tissues. Chen *et al* (2003) and Berendse *et al* (2003) showed reduced gene expression of GLN3 in normal muscle tissue that is because of presence of some myoblast cells with properties of stem cells in muscle tissues. Fan *et al* (2006) presented gene expression of GLN3 in normal tissue and renal tumors that this finding may be as a result of increased proliferation of renal epithelial cells. Thus, according to the low expression of survivin gene in normal liver cell, the high expression of the gene in tumor hepatocytes could be used as a target for immunotherapy in MDV infected birds

In conclusion, the results of this study showed that survivin gene is significantly expressed as an inhibitor of apoptosis gene in tumors with MDV origin in chickens. Immunotherapy of this protein creates a new perspective for the treatment of Marek's disease in valuable birds.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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