INTRODUCTION

Manganese superoxide dismutase (MnSOD) is a metalloenzyme that converts superoxide to hydrogen peroxide and quenches the free radicals generated by the electron transport chain [1]. MnSOD plays a key role in protecting cells against oxidative damage [2] and regulating cellular concentrations of reactive oxygen species (ROS). Altered levels of this crucial enzyme play a significant role in disease development including cancer [3] by activating mitogen-activated protein kinase, which stimulates cell proliferation, and enhancing tumor cell migration [3-9]. Several studies have shown that MnSOD enzymatic activity was significantly lower in Val/Val cancer tissue (96.9±22.9) than in benign tissue (255.44±42.7). Conclusion: Breast cancer tumors contain less MnSOD than benign breast samples. Patients with Ala/Val polymorphism had reduced MnSOD activity compared to patients with Val/Val breast cancer. Ala/Val gene polymorphism may be a risk factor associated with more advanced breast cancer stage. MnSOD gene polymorphism Ala/Val may be a risk factor associated with more advanced breast cancer stage, and reduction of MnSOD activity may be a mechanism of the progression of benign to malignant tumors. Further investigations are needed to evaluate the role of MnSOD in breast cancer progression.

Key Words: Benign, Breast, Cancer, MnSOD, Polymorphism
the mitochondria without defense against superoxide radicals [16]. The alanine (Ala) allele of MnSOD allows for efficient transport into the mitochondrial matrix and generates 30% to 40% more active MnSOD protein than the valine (Val) form of the enzyme [18]. The Val allele is partially arrested in the inner mitochondrial membrane, leading to decreased formation of active MnSOD within the mitochondrial matrix [16,17].

Studies have shown a controversial association between MnSOD gene polymorphism and breast cancer, and Mitrunen et al. [6] and Cai et al. [8] reported that the Ala allele increased the risk of breast cancer. On the contrary, Qiu et al. [19] and Eras-Erdogan et al. [20] reported no association. Therefore, it would be interesting to study the correlation between MnSOD gene polymorphism and breast cancer, and Mitrunen et al. [20] reported no association. Therefore, it would be interesting to study the correlation between MnSOD gene polymorphism and its enzymatic activity in benign and malignant human breast tissue, which may clarify the role of MnSOD in breast cancer progression.

METHODS

Sixty-five females attending King Hussein Medical City (Amman) in 2008, Hanizah Hospital (Amman) in 2010 to 2011, and Al-Basheer Hospital in 2011 (Amman) were enrolled in this study. All females were histopathologically diagnosed with malignant or benign breast cancer. All breast cancer cases were staged from I to IV depending on the combination of Tumor, Node, Metastasis classification according to the National Cancer Institute and National Institutes of Health (USA) and revised by the American Joint Committee on Cancer staging system for breast cancer [21].

Seventeen control females were histopathologically proven free of disease. All participants provided informed consent and were interviewed personally by a professional technician. Data were collected about the participants’ medical history and cancer grade and stage before the samples were collected. Biopsies were collected via fine needle aspiration or surgical biopsy by an oncologist.

MnSOD biochemical activity of the breast biopsies was measured from breast tissue homogenate using a modified protocol that was kindly provided by Spitz and Oberley [22]. Breast biopsies were washed in saline immediately after excision to remove excess blood cells, placed in Eppendorf tubes (Promega, Madison, USA) and stored at -60°C. Biopsies were minced on ice in 300 µL of diethylenetriaminepentaacetic acid buffer, homogenized five times for 7 seconds, burst on ice using an Ultra-Turrax Homogenizer (IKA Labortechnik, Staufen, Germany), and centrifuged at 1,000 rpm for 3 minutes. The tissue homogenates were then placed in Eppendorf tubes and stored at -60°C for protein analysis. Protein concentrations (µg/mL) were determined according to the Lowry method.

MnSOD enzymatic activity was then determined using an indirect competitive inhibition assay based on the principle that O2 will reduce an indicator substrate nitroblue tetrazolium (NBT) and superoxide dismutase (SOD) activity will inhibit the reduction rate in a competitive fashion. SOD-mediated inhibition (% inhibition) of the indicator substrate reduction was plotted as a function of the quantity of protein added to the reaction to construct an inhibition curve. The amount of cellular protein that caused 50% maximum inhibition was then calculated and defined as one unit of SOD activity [22].

Dilutions from each homogenate sample or CuZnSOD standard were prepared prior to the assay in 0.05 M phosphate buffer pH 7.8 such that each 100-µL volume added to the assay contained increasing quantities of protein (1-500 µg) of protein for each sample homogenate or (2-500 ng) of protein for the CuZnSOD standard. Inactivation of CuZnSOD by NaCN was determined by incubation of the assay tubes containing 100 µL of sample or standard mixed with 800 µL of final assay solution for at least 45 minutes at room temperature. Xanthine oxidase solution (100 µL) was then added to initiate the reaction starting with the blank tube (containing 100 µL of phosphate buffer instead of sample) to check for xanthine oxidase concentration and make sure that the preparation produced the desired NBT reduction rate (ideally 0.02 abs/min). The reaction was incubated for 1 minute at room temperature and the absorbance at 560 nm was read every 15 seconds for 2.5 minutes. The rates of abs/min for the blank and the samples were used in calculating percent inhibition. An inhibition curve was established for each sample and standard by plotting of the percent inhibition (%) versus protein quantities (µg), while the maximum inhibition was determined for each curve and used to calculate MnSOD activity units defined as the amount of protein that inhibited NBT reduction by 50% of the maximum inhibition. Finally, units of total MnSOD activity per milligram of protein were calculated for the tissue homogenate samples by taking 1,000 µg of protein per unit.

Genomic DNA was extracted from all blood samples using a Wizard Genomic DNA Purification Kit (Promega, Madison, USA) and stored at -60°C until use. MnSOD gene polymorphism was determined as reported previously [8] using the polymerase chain reaction (PCR) and restriction fragment length polymorphisms method. The PCR product was digested with the NgoMIV restriction enzyme and separated on a 4% agarose gel. The genotypes were determined as heterozygous Ala/Val (18 bp, 89 bp, and 107 bp) bands, homozygous Val/Val (107 bp) band and homozygous Ala/Ala (18 bp and 89 bp). Statistical analysis was carried out using the SPSS version 10.05 (SPSS Inc., Chicago, USA). Means and standard errors of the mean (SE) were calculated and compared among study samples.
**RESULTS**

MnSOD concentrations from the breast tissue homogenates were determined from 65 breast cancer biopsies, 46 benign tissue samples, and 17 controls. MnSOD biochemical activity from benign tissue (236.18 ± 46.37) was higher but not statistically different ($p = 0.087$) from that of cancer tissue (79.83 ± 42.14) as shown in Table 1.

The association among MnSOD biochemical activity in patients with breast cancer, those with benign tissue, and MnSOD genotype was evaluated. There were significant differences in MnSOD activity between the patients with breast cancer (96.9 ± 22.9) and those with benign tissue (255.44 ± 42.7) with Val/Val genotype ($p = 0.045$) as presented in Table 1. Patients with breast cancer who had the Val/Val genotype had significantly higher MnSOD activity (96.19 ± 22.9) than patients with breast cancer who had the Ala/Val genotype (39.19 ± 7.33) ($p = 0.005$). No statistical analysis was conducted for the Ala/Ala genotype due to the absence of this genotype in patients with either breast cancer or benign tissue.

Table 2 shows that the highest frequency (44.2%) of the Ala/Val genotype was associated with advanced invasive cancer (stage III), while the highest frequency of the Val/Val genotype (39.1%) was seen in early cancer (stage II).

**DISCUSSION**

Malignant breast cancer and benign disorders are common diseases among women that cause serious physical and emotional problems for patients and their families. Evidence indicates that mitochondrial oxidative stress may be involved in the development of breast cancer primarily through MnSOD, the major site of ROS production [23], which decreases in metastatic esophageal [24], glioblastoma [25], and other cancers.

To our knowledge, this is the first study that measures MnSOD enzyme activity in human representative mammalian tissues with malignant and benign characteristics. Studies have used human erythrocytes [23], human mammalian cell lines [9], fixed breast tissue samples [26], and human hepatoma cell lines [23]. This study showed that MnSOD activity was lower in cancerous tissues than in benign tissues with the Val/Val phenotype (Table 1), supporting that the MnSOD gene is a tumor suppressor gene [26]. The mechanism responsible for this reduction remains unclear, although it may be attributed to an epigenetic silencing effect of MnSOD [27].

Interestingly, there was a statistically significant difference in MnSOD activity between Val/Val and Ala/Val MnSOD gene polymorphism among cancerous tissue samples only. The Ala-variant genotype confers lower MnSOD activity than the Val-variant, which supports the hypothesis described elsewhere [17] that the transport of the Val-variant of MnSOD may be partially arrested in the inner mitochondrial membrane, while...
the Ala-variant of the MnSOD successfully targets the mitochondria. Ala/Val disrupts the proper targets on MnSOD from the cytosol to the mitochondrial matrix, where it acts on highly reactive superoxide radicals to dismutate it to hydrogen peroxide. Therefore, the change in the level of highly reactive superoxide radicals to dismutate it to hydrogen peroxide in mitochondria to the mitochondrial matrix, where it acts on highly reactive superoxide radicals to dismutate it to hydrogen peroxide.

Patients with the Ala/Val genotype variant conferred low levels of MnSOD activity and were at increased risk of advanced cancer stage compared to the Val/Val variant. The highest frequency of the Val/Ala variant was seen in advanced invasive stage III (Table 2), while the highest frequency of the Val/Val variant was seen in early invasive stage II. These results were similar to those observed by Kucukergin et al. [29]. Furthermore, these results supported that such oxidative stress could be involved in the development and progression of breast cancer since benign tumors have higher MnSOD levels than do malignant tumors, so if MnSOD activity decreased in benign tumors, oxidative damage would accumulate and, consequently, benign tumors might progress into malignant tumors.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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