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# Role of Genetic Predisposition and HPV Genotype in Cervical Cancer in Saudi Arabia

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**Reham M.Alahmadi[1], Fahad N. Al-Majhdi[1] and Ghazi A. Alsbeih[2]**

*[1]Department of Botany and Microbiology, College of Science, King Saud University, P. O. Box – 22452, Riyadh, 11495,Kingdom of Saudia Arabia.*

*[2]King Faisal Specialist Hospital & Research Centre Biomedical Physics Research, Riyadh, 11495, Kingdom of Saudia Arabia .*

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**ABSTRACT**

Cervical cancer is the second most common cancer among women worldwide and persistent infection with human Papillomavirus (HPV) has been identified as a main risk factor for its development. It has been documented that Muslim women have lower incidences of cervical cancer and/or HPV infection raising the question of important environmental, cultural and genetic differences with western countries. Large studies on cervical cancer in Saudi Arabia and informative data are still lacking. This study had investigated the relationship between HPV infection, genotype and the presence of single nucleotide polymorphisms (SNPs) in certain genes involved in p53 and pRb pathways (p53 G72C, p21 C31A, RB1 C525G, CCND1 G870A, and MDM2 promoter T309G) with the occurrence of cervical cancer. Genetic polymorphisms of sixty cervical cancer patients (cases) were compared and assessed with 100 normal volunteers of age-matched women with controls. PCR-based test enables the concurrent detection and genotyping of 37 most common high- and low-risk HPV genotypes. Cancer stage ranged between IA1 and IVA for patients age ranged between 32 and 106 years old (median = 46). By histology, 41 (68.3%) had squamous cell carcinoma and 19 (31.6%) had adenocarcinoma. Most patients had multiple pregnancies (median = 8) of the 60 cervical cancer specimens, 42 samples (70%) were positive for HPV infection. Eight different HPV genotypes were detected: 16, 18, 31, 33, 45, 52, 59, and 73, all of them are classified as high-risk HPV. The prevalence of each HPV genotype among positive patients was: HPV-16 (64.2%), HPV-18 (9.5%), HPV-31 (7.1%), HPV-45 (4.8%), HPV-59 (2.4%) and HPV-73 (2.4%) with HPV-16/45 (4.8%), HPV-16/31 and HPV-33/52 (2.4% each) co-infections. The genotype and allelic distributions of the assessed polymorphisms in the selected genes (p53 G72C, p21 C31A, CCND1 G870A, and MDM2 T309G) were compared between the cancer patients (60 cases) and the volunteers (100 controls – no cancers). Interestingly, the distributions of these SNPs were comparable between cases and controls in this small cohort. Consequently, there were no statistically significant differences for all alleles (P>0.05) suggesting that these SNPs are not involved in predisposition to cervical cancer in Saudi women. Hardy-Weinberg Equilibrium (HWE) tests showed no differences between expected and observed genotypes counts of these SNPs, for both cancer and control cases, suggesting that they are all in equilibrium. In conclusions, 70% of cervical cancer patients were associated with HPV infection. The two most common HPV genotypes were 16 and 18 that formed together 80.96% of HPV infections and 56.7% of all cervical cancer patients. Therefore, current HPV vaccines are expected to provide protection for more than half of cervical cancer women in Saudi Arabia. Genetic predisposition data did not reveal any association between HPV infection and the studied SNPs. Therefore, these SNPs cannot be used as genetic biomarkers to screen individuals who are at high cancer risk to develop cervical cancer. These data provide important baseline information for HPV infection in cervical cancer that can paves the way for future applications of screening and prevention programs of this disease in the Kingdom of Saudi Arabia.

## INTRODUCTION

Cervical cancer is an important health concern for women in the developing countries (Parkin *et al.* 2005) and is the second most common cancer in women worldwide (Nunobiki *et al.* 2010). It is a common cause of death among middle-aged (40-60 years old) women, with an estimated 493,000 new cases and 274,000 deaths worldwide in 2002. It is widely accepted that specific human Papillomavirus (HPV) types are the central etiologic agent of cervical carcinogenesis manifestations. However, other environmental and host factors also play decisive roles in the persistence of HPV infection leading to malignant conversion of cervical epithelium (Nunobiki et al. 2010).

Data concerning the role of genetic polymorphic variations, HPV genotypes, and the development of cervical carcinoma are lacking in Saudi cancer patients. The purpose of this project is to illustrate the association between the human papillomavirus as causative agent, genetic predisposition and cervical cancer in our community. The study included 100 patients with cervical cancer. Archival tumor tissues were examined for HPV infection, genotype and the presence of p53 G72C, p21 C31A, RB1 C525G, CCND1 G870A, and MDM2 T309G genetic polymorphisms presumed to affect cancer susceptibility in conjunction with HPV infection. The distribution of genetic polymorphisms in the general population was evaluated in 100 age-matched women without any history of cancer.

The worldwide HPV prevalence in cervical cancer was estimated at 99.7% (Walboomers *et al.* 1999). In developed countries, the widespread use of Papanicolaou (Pap) smear has dramatically reduced cervical cancer incidence and mortality. Today majority of the cases (83%) occur in developing countries, which is primarily related to the lack of intensive screening programs (Parkin *et al*. 2005).

Polymorphisms are the most common genetic variations between individuals. It has been estimated that each person would harbor 24,000-40,000 non-synonymous SNPs of a total 100,000-300,000 constituting about 1% of the total SNPs in the human genome (Cargill *et al.* 1999). Single-nucleotide polymorphisms (SNPs) are being intensively studied to understand the biological basis of different complex traits conditions and diseases. The hypothesis is that SNPs can influence the rate of mRNA transcription, mRNA stability, its rate of translation to protein and/or the protein-protein interactions. This may cause sub-optimum protein function leading to different degrees of susceptibility to environmental factors, infectious agents and diseases. SNPs located in coding and regulatory regions, such as SNPs in promoter regions

that can alter affinity to transcription factors and non-synonymous SNPs in coding regions that change amino acid sequence, are more likely to be functionally significant and have higher probability to alter protein function. Genetic polymorphic variations in genes involved in cell–cycle control, DNA repair and apoptosis pathways are likely to affect cancer susceptibility. In connection with HPV infection, the p53 and the RB pathways are the two major tumor suppressor pathways that control cell proliferation and senescence. The molecules involved in these two pathways are commonly abrogated in human malignancies including cervical cancer.

It has been suggested that Muslim women, in general, are less susceptible to HPV infection or cervical cancer (Altaf 2001; Bhurgri *et al.* 1999; Jamal and Al-Maghrabi 2003; WHO/ICO 2010). The incidence of cervical carcinoma is very low in Saudi women. According to the Saudi National Cancer Registry 2007 Report (Ministry of Health 2007), cancer of the cervix is the fifteenth most common malignancy in Saudi females, forming 2.2% of newly diagnosed cancer cases. The closer society and standards of mores could play a role, but the actual reasons for the low incidence remain unclear. Nonetheless, it forms 3.1% of all cancers in Saudi women in the most active age (30-59 years), ranking as the fifth most common cancer in age-group (30-44) and the seventh in age-group (45-59). In addition, the awareness of cervical cancer screening and its underlying etiology and preventive measures among women in Saudi Arabia are poor and far behind when compared with developed countries (Sait 2009).

Data on HPV infection among Muslim women worldwide is scanty. In general, the low prevalence of cervical cancer in Muslim and in Jewish women could suggest low HPV infection (Altaf 2001; Arbel-Alon *et al.* 2002; Castellsague *et al.* 2002). However, preliminary reports from countries with large Muslim populations are controversial (de Boer *et al.* 2006; Duttagupta *et al.* 2004). For example, Indonesia, the largest Muslim population, has high rate of HPV infection and cervical cancer (de Boer et al. 2006). To our knowledge, there is little data on HPV infection in Saudi Arabia. Studies profiling Pap smears has pointed out that the precursor lesions of cervical cancer are present in Saudi women (Jamal and Al-Maghrabi 2003; Mansoor 2003), and the infection with HPV 16/18 genotype was present in one-third of the patients (Al-Muammar *et al.* 2007). However, the full picture of the association between HPV infection, the presence of the various HPV genotypes and cervical cancer in Saudi women remains unknown.

The study approached to identify the frequency of the following SNPs: p53 G72C, p21 C31A, RB1 C525G, CCND1 G870A, and MDM2 T309G, in cervical cancer patients. Also, this work determines HPV infection and genotypes in invasive cervical cancer patients and the association between the studied SNPs, HPV infection.

## MATERIALS AND METHODS:

The study population included 60 patients (*n=60*) with histopathologically proven cervical cancer. The participation of the patients was limited to providing paraffin embedded tissues that were obtained from the Department of Pathology and Laboratory Medicine at King Faisal Specialist Hospital and Research Centre. There are no restrictions on patients' age or histological type of cervix cancer (whether squamous cell carcinoma or adenocarcinoma). Controls (normal volunteers, ***NV***, *n=100*) were randomly selected from healthy women with no history of cancer. All women were Muslims.

### Cell Lysis

Fifteen ml of RBC Lysis Solution (Cat. No. 158904, Qiagen, USA) was added into the 50 ml tube containing the thawed whole blood cells. The tube was inverted several times to mix and was incubated for 5 minutes at room temperature. Samples were centrifuged for 5 minutes at the speed of 4000 rpm. After which, the supernatant was removed leaving behind the visible white pellet and about 200-400 µl of the residual liquid. All tubes were vortexed vigorously to re-suspend the white pellets in the residual supernatant and this greatly facilitates cell lysis for the following step. Five ml of Cell Lysis Solution (Cat. No. 1589, Qiagen, USA) was added to the tube and solution was pipetted up and down to lyse the cells. If the solution is not homogeneous, incubation at $37^0$C or room temperature was needed until the solution becomes homogeneous. Samples were stable in Cell Lysis Solution for at least 2 years at room temperature.

### *RNAse* Treatment

*RNase A* Solution (25µl of 4 mg/mL, Cat. No. D50K6, Qiagen, USA) was added to the cell lysate. The samples were mixed by inverting the tube 25 times and incubated at $37^0$C for 15 minutes.

**DNA and Protein Precipitation**

Sample was then cooled to room temperature, then 1.67 ml of Protein Precipitation Solution (Cat. No. 158912, Qiagen, USA) was added to the cell lysate. The sample was vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate. Subsequently, the sample was centrifuged at 4000 rpm for 5 minutes to precipitate the protein. The precipitated proteins then formed a tight, dark brown pellet.

The supernatant containing the DNA was carefully poured off (leaving behind the precipitated protein pellet) into a clean 50 ml tube, containing 5 ml of 100% Isopropanol. The tube containing the supernatant liquid was mixed by inverting 50 times until white threads of DNA formed a visible clump. Tube was centrifuged at 4000 rpm for 3 minutes; the DNA became visible as a small white pellet. Immediately after, 5 ml of 70% Ethanol was added to the tube and inverted for several times to wash the DNA pellet. Sample was centrifuged again at 4000 rpm for 1 minute and later on, the ethanol was carefully poured off without losing the DNA pellet. Thereafter, the tube was drained and the sample was allowed to air dry for 10-15 minutes.

**DNA Hydration**

Five hundred µl of the DNA Hydration Solution, (Tris [hydroxymethyl] aminomethane & ethylenediaminetetraacetic acid, Cat. No. 158916, Qiagen, USA), was added to the DNA pellet. DNA was rehydrated by incubating at $65^0$C for 1 hour, and then the tube was vortexed and incubated overnight at room temperature. The tube was tapped periodically to aid in dispersing the DNA. Extracted DNA was centrifuged briefly and then transferred to a 1.5 ml tube and stored at $4^0$C. For long term storage, the DNA was kept at $–20^0$C or $–80^0$C.

DNA was extracted from paraffin-embedded tissue samples by using QlAmp DNA FFPE tissue Kit (50) from Qiagen, USA (Cat. No.56404). For each sample, 3 sections of 10-micrometer were cut from the block for DNA analysis. The sections were immediately placed in a 1.5 or 2ml microcentrifuge tube and 1ml xylene was added to the sample, then vortexed vigorously for 10 seconds. Samples were centrifuged at full speed for 2 minutes at room temperature. The supernatant was removed by pipetting carefully without removing any of the pellets. Afterward, 1ml of ethanol (96-100%) was added to the pellet and mixed by vortexing to extract xylene from the sample. The sample was centrifuged at full speed for 2

min at room temperature. The supernatant was removed by pipetting without removing any of the pellets.

The tube was incubated at $37^0$C for 10min until all residual ethanol has evaporated. The pellet was resuspended in 180µl buffer ATL. Then 20µl proteinase K was added and mixed by vortexing. The sample was then incubated at $56^0$C for 1h (or until the sample has been completely lysed), and then at $90^0$C for 1hr. Two hundred (200)µl buffer AL was added to the sample and mixed thoroughly by vortexing. Then 200 µl ethanol (96- 100%) was then added and sample was mixed again. Afterward, the 1.5 ml tube was centrifuged briefly to remove drops from the inside lid. The entire lysate was carefully transferred to the QlAmp MinElute column (in a 2 ml collection tube) without wetting the rim, then the lid was closed and centrifuged at 8000 rpm for 1 min. The QlAmp MinElute column was placed in a clean 2 ml collection tube and the collection tube containing the flow-through was discarded.

Five hundred µl buffer AW1 was added without wetting the rim of the QlAmp MinElute column. The samples were centrifuged at 8000 rpm for 1 min. The QlAmp MinElute column was then placed in a clean 2 ml collection and the collection tube containing the flow-through was discarded. Then, 500 µl buffer AW2 was added without wetting the rim. Samples were centrifuged again at 8000 rpm for 1 min. Each sample was placed in a clean 2 ml collection tube, and the collection tube containing the flow-through was discarded again. The tube was centrifuged at full speed (14,000 rpm) for 3 min to drain the membrane completely. The QlAmp MinElute column was placed in a clean 1.5ml microcentrifuge tube then the collection tube containing the flow-through was discarded. Then, 20-100 µl Buffer ATE was added to the center of the membrane. Immediately after, the tube was incubated at room temperature (15 - 25 $^0$C) for 60 min and then centrifuged at full speed (14,000 rpm) for 1 min.

**HPV Detection and Genotyping**

HPV detection and genotyping had been carried out using the newly introduced Linear Array Detection Kit and Linear Array HPV Genotyping Test (Roche Diagnostics). This PCR-based test enables the concurrent detection and genotyping of 37 most common high- and low-risk HPV genotypes, including those considered as significant risk factor for progression to cervical cancer. The HPV high risks genotypes are: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. While, The HPV low risks genotypes are: 6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108.

The test has the beta-globin gene as an internal control to show adequacy of the sample and it also enables detection of multiple or double HPV infections. The primers and the PCR reaction conditions are provided with the test. The manufacturer recommended that the methodology must be followed strictly.

**PCR Amplification**

Linear Array HPV Genotyping Test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 base pairs long. A pool of HPV primers present in the master mix is designed to amplify HPV DNA from 37 HPV genotypes including 13 high-risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68). Captured probe sequences are located in polymorphic regions of L1 bound by these primers. An addition primer pair targets the human β-globin gene to provide a control for cell adequacy, extraction and amplification.

AmpliTaq gold *DNA polymerase* was utilized for 'hot start' amplification of the HPV target DNA and the control. First, the PCR reaction mixture was heated to activate AmpliTaq Gold DNA polymerase to denature the viral DNA and human genomic DNA to expose the primer target sequences. As the mixture cools, the primers (both upstream and downstream) anneal to the target DNA. The AmpliTaq gold *DNA polymerase*, in the presence of $Mg^{2+}$ and excess dNTPs, extends the annealed primers along the target templates to produce an approximately 450 base pair double-stranded HPV target DNA molecule or a 268 base pair β-globin DNA molecule termed an amplicon. This process was repeated for a number of cycles, each cycle effectively doubling the amount of amplicon DNA. Amplification occurs only in the region of the HPV genome or β-globin gene between the appropriate primer pair. The entire genome is not amplified.

Selective amplification of the target nucleic acid from the specimen is achieved in the Linear Array HPV Genotyping Test by the use of *AmpErase (uracil-N-glycosylase)* enzyme and deoxyuridine triphosphate (dUTP). *AmpErase* enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine, but not DNA containing deoxythymidine. Deoxuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate in addition to deoxthymidine triphosphate in the master mix reagent; therefore only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by *AmpErase* enzyme prior to amplification of the target DNA. *AmpErase* enzyme, which is included in the

master mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position when heated in the first thermal cycling step at the alkaline pH of master mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. *AmpErase* enzyme is inactive at temperatures above 55˚C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon. Following amplification, any residual enzyme is denatured by the addition of the denaturation solution, thereby preventing the degradation of any target amplicon.

**Hybridization Reaction**

Following PCR amplification, the HPV and the β-globin amplicons are chemically denatured to form single-stranded DNA by the addition of denaturation solution. Aliquots of denatured amplicon are then transferred to a well that contains hybridization buffer and a single linear array HPV genotyping strip (strip) that is coated with HPV and β-globin probe lines. The biotin-labeled amplicon will hybridize to the oligonucleotide probes only if the amplicon contains the matching sequence of the complementary probe. In addition, the strip is coated with one cross-reactive oligonucleotide probe that hybridizes with HPV genotypes 33, 35, 52 and 58. Amplicon containing closely matching sequences (only 1 to 3 mismatches) complementary to the probe will hybridize to this probe line.

**Detection Reaction**

Following the hybridization reaction, the strip is stringently washed to remove any unbound material. Streptavidin-Horseradish peroxidase conjugate is then added to the strip. Streptavidin-Horseradish peroxidase conjugate binds to the biotin-labeled amplicon hybridized to the oligonucleotide probes on the strip. The strip is then washed to remove any unbound streptavidin-Horseradish Peroxidase conjugate and a substrate solution containing hydrogen peroxide and 3,3’,5,5’- tetramethylbenzidine (TMB) is added to each strip. In the presence of hydrogen peroxide, the bound *streptavidin-horse-radish peroxidase* catalyzes the oxidation of TMB to form a blue colored complex, which precipitates at the probe positions where hybridization occurs. The strip is read visually by comparing the pattern of the linear array HPV genotyping Test Reference guide.

**Detection and Genotyping**

All detection reagents were warmed to room temperature. The water bath and shaking water bath were pre-warmed to $53^0$C ± $2^0$C at a shaking speed of approximately 60 RPM. The Working Hybridization Buffer and Working Stringent Wash Buffer were warmed at $53^0$C ± $2^0$C water bath for a minimum of 15 minutes and left in the water bath until use. Working Hybridization Buffer, Working Ambient Wash Buffer and Working Citrate Buffer are sufficient for 60 LINEAR ARRAY HPV Genotyping Strips.

Each HPV strip was labeled with a water/chemical/heat-resistant permanent ink pen with the appropriate specimen or control identification. Each strip was placed upward into the appropriate well of the 24-well tray with the probe lines facing up. Next, 75 µl of denatured amplicon was pipetted into the appropriate well containing a labeled strip. Then, 4 ml of pre-warmed Working Hybridization Buffer was added to each well. The tray was covered with the lid and placed in $53^0$C ± $2^0$C shaking water bath. The amplicons were hybridized for 30 minutes at a shaking speed of approximately 60 rpm. During hybridization, Working Conjugate was prepared as follows: 15µl SA-HRP was added to 5ml of Working Ambient Wash Buffer for each strip that was used. Conjugate was stored at room temperature in a clean container and is stable for only 3 hours.

The tray was removed from the shaking water bath and the Working Hybridization Buffer was removed from the wells by vacuum aspiration. Immediately after, 4 ml of Working Ambient Wash Buffer was added to each well containing a strip. The tray was rocked gently for 3-4 times to rinse the strips. Then, the Working Ambient Wash Buffer was vacuumed from the wells. Four milliliters (4 ml) of pre-warmed Working Stringent Wash Buffer was then $53^0$C ± $2^0$C shaking water bath and incubated for 15 minutes at a shaking speed of approximately 60 rpm. The tray was removed again from the shaking water bath and the Working Stringent Wash Buffer was removed from the wells by vacuum aspiration.

Working Conjugate (4 ml) was added to each well containing a strip and the tray was placed on an orbital shaker incubated for 30 minutes at room temperature. The tray was removed from the orbital shaker and the Working Conjugate was removed from the wells by vacuum aspiration. Working Ambient Wash Buffer (4ml) was again added to each well containing a strip and the tray was rocked gently for 3-4 times to rinse the strips. Immediately after, the buffer was removed. Then, another 4ml of wash buffer was added, the tray was placed on orbital shaker for 10 minutes at a shaking speed of approximately 60 rpm at room

temperature. The tray was then removed from the orbital shaker and the buffer was removed. This step was repeated twice.

Four milliliters (4ml) of Working Citrate Buffer was added and the tray was rocked for 5 minutes. Then the buffer was removed. Afterward, 4ml of Working Substrate was added to each well and the tray was rocked for 5 minutes. The buffer was removed by vacuum aspiration. Finally, 4 ml of distilled or deionized water was added to each well. The strips were removed from the tray using clean forceps, placed on a clean and dry surface and allowed to air dry for a minimum of one hour up to 72 hours at room temperature prior to interpretation.

**DNA Amplification and SNPs Genotyping**

Relevant segments of DNA (50ng/µL) were amplified using PTC-220 DNA Engine Dyed™ Peltier Thermal Cycler (MJ research, USA). Samples were brought to a final volume of 25 microliters. Each reaction contained 0.3µl HotStarTaq DNA Polymerase, 12.5 µl Distilled Water, 2.5µl PCR Buffer, 5µl Q Buffer, 2µl dNTP and 0.4µl of forward and reverse primers (10:90 dilutions) were used to amplify the target DNA. A standard PCR amplification consisted of 39 core cycles of 1 minute denaturing at $95^0$C, 1 minute primers' annealing at the optimized temperature (specifically mentioned for each SNP) and 1 minute primers' extension at 72 $^0$C

The amplified fragment is directly sequenced using the Dynamic ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) according to the manufacturer's instruction. Samples were sent to the sequencing core facility and are run on the MegaBase 1000 sequencer (Applied Biosystems, 3730xl DNA analyzer (96) - Model No. 6250020).
Sequencing results were aligned to the corresponding reference sequence and the SNPs were genotyped using SeqManII sequence analysis software (DNASTAR Inc.).

**Statistical Analysis and Ethical Considerations**

The calculation of the projected sample size (100 patients) is not based on any statistical power calculation but compared to published data (often less than 100) in other populations. However, we could only include 60 patients despite all efforts made to collaborate with other hospitals. The study has been performed on left-over, already acquired pathologic materials and therefore, they are subject to waiver from consent form. The samples were coded and

processed anonymously with no patients' identifiable scripts. One hundred normal volunteers (controls, *n=100*), healthy women with no history of cancer, were included in the study following signing a consent form (see Appendix I).

For statistical analysis, the correlations between cervical cancer, HPV status, and SNPs are analyzed by two-sided Fisher's exact test. Statistical analyses are performed using the SPSS package software (SPSS Inc., Chicago, IL). The association between cancer occurrence and SNPs genotype and allelic frequencies were measured by the odds ratio (OR) with its confidence interval. The degree of significance was calculated using the Chi-Squares method. A p-value of 0.05 or less was considered statistically significant. Furthermore, Hardy-Weinberg equilibrium (HWE) and deviation from HWE for genotype distribution were tested for each SNP to study the disturbing influences of genotype frequency in the population by comparing the observed to the expected genotypes' frequencies.

## RESULTS

### Clinical Data

The age at diagnosis of the 60 patients included in the study ranged between 32 and 106, with a median of 46 years old. By histology, 41 (68.3%) patients had squamous cell carcinoma while 19 (31.6%) had adenocarcinoma of the cervix. The distribution of these two histopathological types by 10-year age groups is given in Figure 1. Beside 1 single woman, most patients had multiple pregnancies with a median number of live births of 8. The stage of the disease ranged between IA1 and IVA.

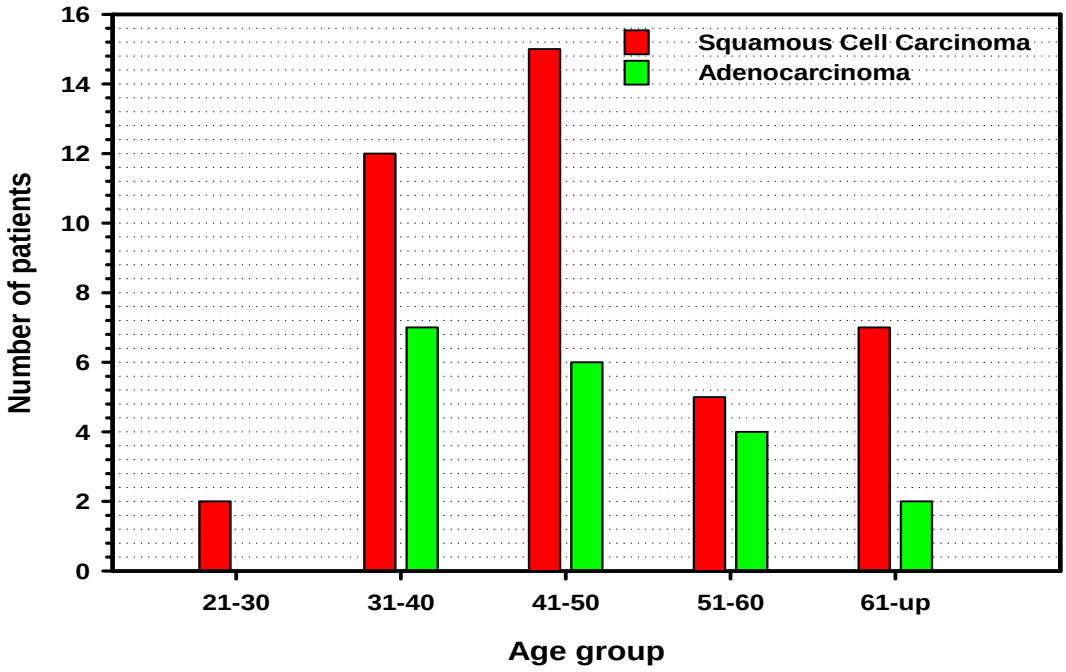



**Figure 1. The distribution of squamous cell carcinoma and adenocarcinoma by 10-year age group in the 60 cervical cancer patients**.

**HPV Detection and Genotyping**

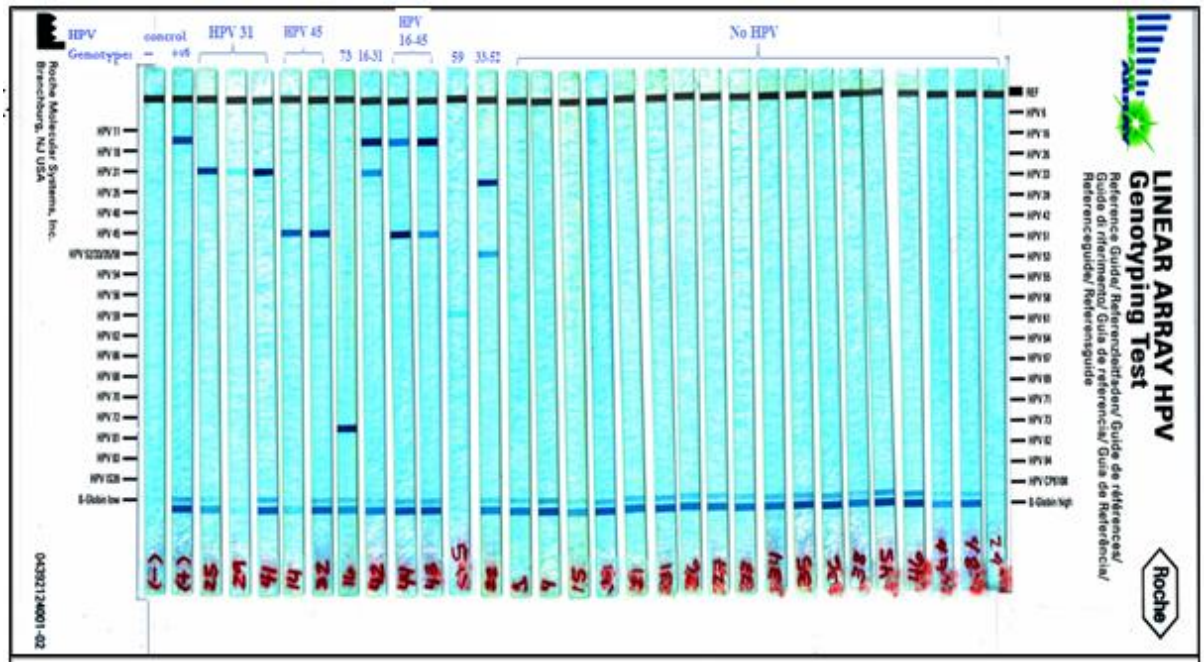
Linear Array HPV detection and genotyping were carried out in 6 separate batches of 12 strips as recommended by the manufacturer. Each batch has 2 internal controls: negative control of no DNA to check for the absence of DNA contamination, and positive control for HPV-16 genotype (Figure 2). Successful test or presence of DNA is judged by the appearance of 2 β-globin blue bands. Negative results have only the 2 β-globin bands while positive results have in addition one or more bands corresponding to the HPV genotypes. Of the 60 cervical cancer specimens, 42 samples (70%) were positive for HPV infection. In total, 8 different HPV genotypes were detected all of them (16, 18, 31, 33, 45, 52, 59, and 73) are commonly classified as high-risk HPV. Thus, there were no low-risk HPV genotypes detected in this series of 60 cervical cancer patients. The prevalence of each HPV genotype among positive patients is listed in Table 1.

**Table 1. Prevalence of different HPV genotypes in 42 HPV-positive cervical cancer patients.**

| HPV genotypes | Classification | Number of patients | Prevalence (%) |
|---|---|---|---|
| *Single Infection* | | | |
| HPV-16 | HR | 27 | 64.2 |
| HPV-18 | HR | 4 | 9.5 |
| HPV-31 | HR | 3 | 7.1 |
| HPV 45 | HR | 2 | 4.8 |
| HPV59 | HR | 1 | 2.4 |
| HPV73 | HR | 1 | 2.4 |
| *Co-Infections* | | | |
| HPV-16/31 | HR/ HR | 1 | 2.4 |
| HPV-16/45 | HR/ HR | 2 | 4.8 |
| HPV-33/52 | HR/ HR | 1 | 2.4 |

LR: low risk; HR: high risk.

**(A)**



**(B)**

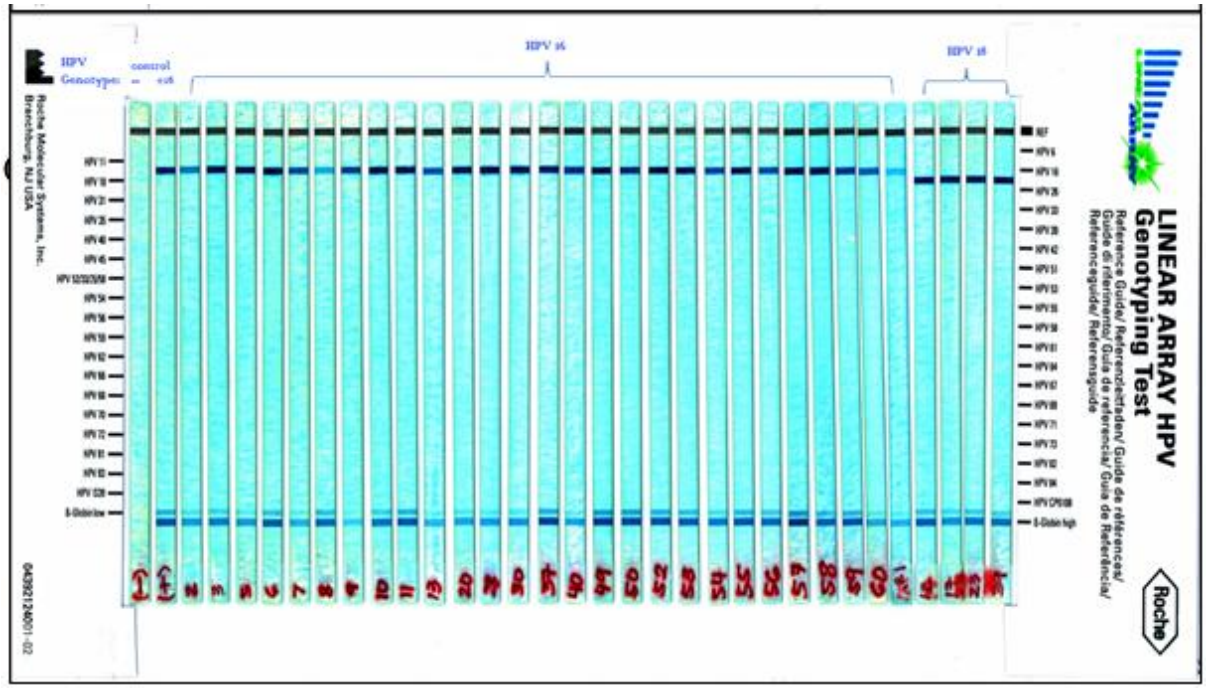



**Figure 2: (A and B). HPV results obtained using the Linear Array HPV Genotyping Test (Roche Diagnostics), showing negative and positive controls, and the different HPV genotypes in single and double infections detected in the 60 cervical cancer patients.**

The results showed that the most common HPV genotypes with single infection were: HPV-16 (64.2% of patients), HPV-18 (9.5%), HPV-31 (7.1%) and HPV-45 (4.8%). The HPV-59 and HPV-73 had only affected one patient each (2.4%). Furthermore, HPV-16/45 (4.8%), HPV-16/31 and HPV-33/52 (2.4% each) co-infections were also present (Table 1). The age distribution of various HPV genotypes is shown in (Figure 3). Considering single infection, the two most common genotypes were HPV-16 and HPV-18. These two genotypes affected together 34 patients, i.e. 80.96% of all HPV-positive patients, and were equally frequent in young (≤46) and old (>46) age groups (80% compared to 81%, respectively).

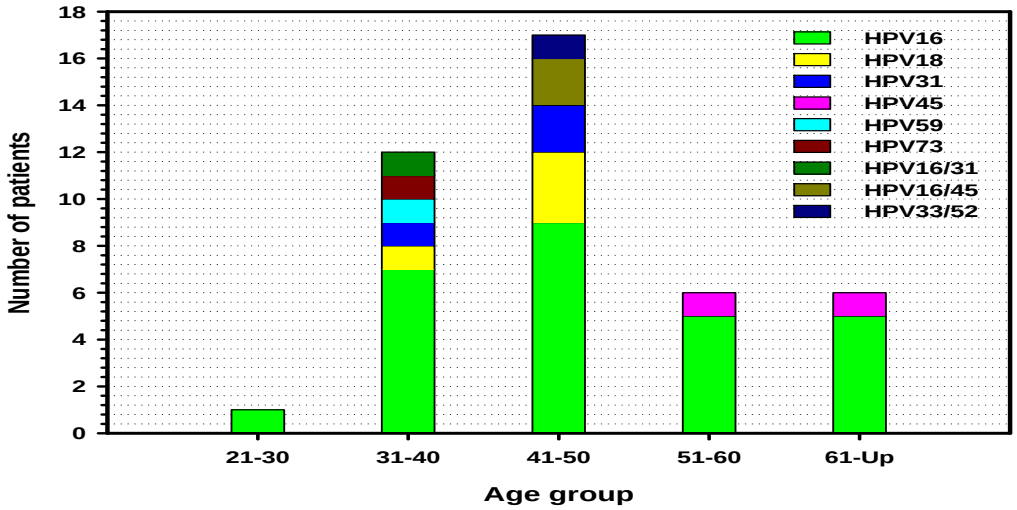



**Figure 3. The distribution of HPV genotypes by 10-year age group in 42 HPV-positive cervical cancer patients.**

**Distribution of Genetic Polymorphic Variations**

The genotype and allelic distributions of the assessed polymorphisms in the selected genes for the 60 cervical cancer patients (cases) and the 100 normal volunteers (controls) are listed in Table 2 (see figure 6 A, B, D, C). The three genotypes; homozygous wild-type, heterozygous and homozygous variants were observed for p53 G72C, p21 C31A, CCND1 G870A, and MDM2 promoter T309G in this cohort of Saudi individuals. Unfortunately, although PCR conditions for RB1 C525G have worked well in optimization experiments, no results were obtained for the patients' and controls' samples despite repeating primers designs and multiple modifications in PCR conditions. This was attributed to the nature of the highly repetitive sequence in this DNA region of RB1. Therefore, this polymorphism has been withdrawn from this study due to time constraint to complete the thesis. The frequencies of the remaining SNPs studied were compared between the cancer patients (cases) and the volunteers (controls – no cancers). Interestingly, the distributions of these SNPs were comparable between cases and controls in this small cohort. Consequently, there were no statistically significant differences for all alleles ($P>0.05$) suggesting that these SNPs are not involved in predisposition to cervical cancer in this cohort, although it should be further investigated and researched in larger population to confirm of refute these results. The current data reveal negative association between HPV infection and the chosen SNPs in this study.

**Hardy-Weinberg Equilibrium (HWE)**

For HWE tests, the 5% significance level for 1 degree of freedom is 3.84. Interestingly, none of the chi-squared values of the differences between expected and observed genotypes' counts of p53 G72C, p21 C31A, CCND1 G870A, and MDM2 promoter T309G for both cancer and control cases were equal to or greater than 3.84 suggesting that they are all in the Hardy–Weinberg equilibrium (Table 3). Furthermore, the frequencies observed in Saudi Arabia of all SNPs studied were comparable to those available at the National Center for Biotechnology Information Website (NCBI).

**Table 2. Genotypes and alleles frequencies of 4 assessed SNPs in a cohort of 160 Saudi individuals (60 with cervix cancer and 100 controls without cancer)**

| Gene, genotype and allele | Total Samples: 160 Cases n=60(%) | Controls n=100(%) | Odds Ratio CI 95% | P-value | Overall frequencies (%) | *NCBI frequencies (%) |
|---|---|---|---|---|---|---|
| **p21 (CDKN1A) codon 31 C>A Ser/Arg Rs1801270** | | | | | | |
| **C/C** | 40(69) | 64(64) | | | 65 | 55 |
| **C/A** | 15(16) | 33(20) | 0.73 (0.35-1.50) | NS[C] | 30 | 32 |
| **A/A** | 3(14) | 3(8) | 1.60(0.31-8.32) | NS[C] | 4 | 13 |
| **C** | 95(82) | 161(81) | | | 80 | 71 |
| **A** | 21(18) | 39(20) | 0.91(0.51-1.64) | NS[C] | 18.75 | 29 |
| **p53 (TP53) codon 72 G>C Arg/Pro Rs1042522** | | | | | | |
| **G/G** | 11 (19) | 21 (21) | | | 20 | 36 |
| **G/C** | 33 (60) | 53 (56) | 1.19(0.51-2.78) | NS[C] | 53.75 | 41 |
| **C/C** | 13 (22) | 26 (25) | 0.95(0.35-2.56) | NS[C] | 24.375 | 23 |
| **G** | 55 (48) | 95 (48) | | | 46.875 | 56 |
| **C** | 59(52) | 105(53) | 0.97(0.61-1.54) | NS[C] | 51.25 | 44 |
| **CCND1 codon 870 G>A Pro/Pro RS9344** | | | | | | |
| **G/G** | 13 (24) | 34 (34) | | | 29.375 | 17 |
| **G/A** | 33 (56) | 43 (39) | 2.01(0.92-4.39) | NS[C] | 47.5 | 43 |
| **A/A** | 9 (18) | 23 (26) | 1.02(0.38-2.78) | NS[C] | 20 | 41 |
| **G** | 59 (54) | 111 (56) | | | 53.125 | 38 |
| **A** | 51 (46) | 89 (45) | 1.08(0.67-1.72) | NS[C] | 43.75 | 62 |
| **MDM2 promoter 309 T>G rs2279744** | | | | | | |
| **T/T** | 16 (29) | 33 (33) | | | 30.625 | N/A |

| | | | | | | |
|---|---|---|---|---|---|---|
| **T/G** | 27 (46) | 49 (43) | 1.14(0.53-2.43) | NS[C] | 47.5 | N/A |
| **G/G** | 12 (24) | 18 (21) | 1.38(0.53-3.53) | NS[C] | 18.75 | N/A |
| **T** | 59 (54) | 115(58) | | | 54.375 | N/A |
| **G** | 51 (46) | 85 (43) | 1.17(0.73-1.87) | NS[C] | 42.5 | N/A |

*Manually calculated from NCBI website for each SNP.

[c]NS: not significant (P>0.05)

**Table 3: Test for Hardy-Weinberg equilibrium (HWE) and deviation from HWE for SNPs significantly associated with cervix cancer occurrence.**

| | **Genotypes** | | | **In HWE[a]** | **Significance level** | **Deviation from HWE[a]** | **Significance level** |
|---|---|---|---|---|---|---|---|
| | **Wild-type** | **heterozygote** | **Variants** | **Chi-Square[b]** | **P** | **Chi-Square[b]** | **p** |
| **Gene** | p21 (CDKN1A) codon 31 C>A Ser/Arg | | | | | | |
| | Cancer patients | | | | | | |
| **Observed #** | 40 | 15 | 3 | 0.95 | NS[C] | 0.33 | NS[C] |
| **Expected #** | 38.9 | 17.2 | 1.9 | | | | |
| | Control individuals | | | | | | |
| **Observed #** | 64 | 33 | 3 | 0.26 | NS[C] | 0.61 | NS[C] |
| **Expected #** | 64.8 | 31.4 | 3.8 | | | | |
| **Gene** | p53 (TP53) codon 72 G>C Arg/Pro | | | | | | |
| | Cancer patients | | | | | | |
| **Observed #** | 11 | 33 | 13 | 1.45 | NS[C] | 0.23 | NS[C] |
| **Expected #** | 13.27 | 28.46 | 15.27 | | | | |
| | Control individuals | | | | | | |
| **Observed #** | 21 | 53 | 26 | 0.39 | NS[C] | 0.53 | NS[C] |
| **Expected #** | 22.56 | 49.88 | 27.56 | | | | |
| **Gene** | CCND1 | | | | | | |
| | Cancer patients | | | | | | |
| **Observed #** | 13 | 33 | 9 | 2.34 | NS[C] | 0.125 | NS[C] |
| **Expected #** | 15.82 | 27.35 | 11.82 | | | | |
| | Control individuals | | | | | | |
| **Observed #** | 34 | 43 | 23 | 1.68 | NS[C] | 0.195 | NS[C] |
| **Expected #** | 30.8 | 49.4 | 19.8 | | | | |
| **Gene** | MDM2 promoter 309 T>G | | | | | | |
| | Cancer patients | | | | | | |
| **Observed #** | 16 | 27 | 12 | 0.01 | NS[C] | 0.92 | NS[C] |
| **Expected #** | 15.82 | 27.35 | 11.82 | | | | |
| | Control individuals | | | | | | |
| **Observed #** | 33 | 49 | 18 | 0 | NS[C] | 0.98 | NS[C] |
| **Expected #** | 33.06 | 48.87 | 18.06 | | | | |

[a] HWE: Hardy-Winberg Equilibrium

Pearson's chi-square at 1 degree of freedom

[c]NS: not significant ( the 5% significance level for 1 degree of freedom is ≥ 3.84)

## DISCUSSION

Cervical cancer is the second most common cancer in women worldwide, and preventable and curable disease especially if identified at an early stage. It is widely accepted that specific HPV types are the central etiologic agent of cervical cancer development. Other environmental and host factors also play decisive roles in the persistence of HPV infection and further malignant transformation. Although many previous reports have focused on HPV environmental factors, the role of host susceptibility to cervical carcinogenesis is still largely unknown (Nunobiki *et al.* 2011).

Cervical cancer and its precursors have been associated with several epidemiological variables. The risk factors for cervical cancer includes young age at first sexual intercourse (<20 years old), multiple sexual partners, sexual partner with multiple sexual partners, young age at first pregnancy, high parity, lower socioeconomic status and smoking. These risk factors basically increase the likelihood of exposure to high-risk HPV types. The disease is relatively rare before 20 years of age and the mean age is about 47 years old.

In this study including 60 cervical cancer patients, we have conducted HPV detection and genotyping in invasive cervical cancer in the Kingdom of Saudi Arabia. Our data showed that 70% of histopathologically proven cervical tumors were positive for any HPV infection detected by the Linear Array Test Kit (Figure 2). This prevalence of HPV infection in Saudi cervical cancer patients is lower than the estimated 85–99% worldwide and also lower than what was previously reported in Saudi Arabia (Alsbeih et al. 2011; de Sanjose *et al.* 2010).

By histological type (Figure 3), squamous cell carcinoma (n=41) was nearly 2-fold more common than adenocarcinomas (n=19) which is much higher than what has been described previously (Alsbeih et al. 2011). Nevertheless, HPV infection seemed to be an important risk factor for both as it was found in 74% (14/19) of adenocarcinomas and 68% (28/41) of squamous cell carcinomas. Our results showed the presence of 8 different HPV genotypes which (16, 18, 31, 33, 45, 52, 59, and 73) are commonly classified as high-risk and there is no low-risk HPV genotypes in this group of patients. Thus, the prevalence of high-risk genotypes was 100% of HPV-positive tumors which is comparable to the regional results obtained by Darnel *et al.* involving 44 Syrian women with invasive cervical cancer (Alsbeih et al. 2011; Darnel *et al.* 2010).

In agreement with other studies, the most common HPV genotype was HPV-16 (Sjoeborg *et al.* 2010) (Table 1) with a prevalence of 64.2% compared to 54.4% in the world. The following most common genotypes by decreasing prevalence were HPV-18 (9.5%), HPV-31 (7.1%) and HPV-45 (4.8%). The remaining HPV genotypes (59 and 73) have an estimated prevalence of 2.4% each. Co-infections implicated HPV-16/45 (4.8%), HPV-16/31 (2.4%) and HPV-33/52 (2.4%). The two most common genotypes were HPV-16 and HPV-18 with an estimated overall prevalence in cervical cancers of 56.7% (34/60) and 80.96% (34/42) of those who are HPV-positive. This is lower than the overall prevalence in cervical cancer observed in Africa (69.7%), Europe (74.5%), America (70.7%), Asia (68.5%) and in the whole world (70.9%) (WHO/ICO 2010). However, our results seemed to be different from those obtained in another Middle Eastern country where the most common HPV genotype was 33 followed by 16 and 18 (Darnel *et al.* 2010).

Genetic predisposition to cervical cancer is a contributing factor to smoking, environmental and occupational exposures as well as the interaction with oncogenic viral infections (Al-Hadyan *et al.* 2011). In this association study, we have investigated the role of 5 polymorphisms in genes involved in cell cycle and DNA repair pathways as risk factor for cervical cancer occurrence. The SNPs selected were p53, p21, MDM2, CCND1 and RB1 genes. Results on 60 cervical cancer patients and 100 normal volunteers were obtained for the first 4 genes while the latter (RB1) failed to amplify despite repetitive primers designs and PCR conditions. Interestingly, the association study comparing various SNPs genotypes between cervical cancer patients (cases) and normal volunteers (controls), did not show any noticeable differences and therefore, no statistically significant association was found for p53 G72C, p21 C31A, CCND1 G870A, and MDM2 promoter T309G at allele or genotype level. These results suggest that these SNPs are not involved in predisposition to cervical cancer in this cohort of Saudi women.

The p53 is a tumor suppressor gene involved in multiple pathways including apoptosis, cellular transcriptional control, and cell cycle regulation. A large number of human tumors, including smoke-induced lung cancer, show mutations and deletions of the p53 gene that result in loss of tumor suppression function and cell cycle de-regulation. The polymorphism at codon 72 of the p53 gene, which results in the substitution of arginine (Arg) for proline (Pro) in the gene product is common in the general population. It has been suggested that the homozygous Arg genotype increased the susceptibility of p53 protein to degradation by E6 protein derived from oncogenic HPV (Nunobiki *et al.* 2011) and therefore, the “G” allele

would be more frequent in cervical cancer patients. In accordance with this provision, a study by Jiang and colleagues including 104 cervical cancer patients and 160 controls, showed that there is a significant association between the genetic polymorphism of p53, and also p21, and the risk of cervical cancer among Chinese southern women (Jiang *et al.* 2010). However, our results did not show any preponderance of this allele in Saudi cervical cancer patients as compared to the controls. In agreement with our results, a study including 749 cervical cancer patients showed no significant association for p53 (von Keyserling *et al.* 2011).

The p21 protein is induced by p53 in response to DNA damage caused by exposure to environmental carcinogens and acts as cell cycle inhibitor to arrest cell growth to allow DNA repair (Powell *et al.* 2002). Therefore, p21 polymorphism was suggested to affect cancer risk and several studies have correlated the p21 codon 31 SNP with various human malignancies. Results presented here showed that the p21 codon 31 variant allele "A" was not associated with increasing risk to develop cervical cancer in Saudi Arabia. These results are in line with study by Li *et al.* which included 1415 CC cases and 1947 controls that reported no association between the p21 polymorphism Arg31Ser and risk of cervical cancer among Asians (Li *et al.* 2011). Nonetheless, it was suggested that the combination of the Pro allele containing genotypes of p53 and the Ser homozygous genotype of p21 may pose a remarkably increased risk for cervical cancer (Roh *et al.* 2010).

MDM2 is the key negative regulator of p53 and dysfunction of these genes may be associated with an increased rate of accumulation of genetic errors thereby enhancing the progression of the disease. A functional T to G polymorphism in the promoter region of the MDM2 gene (MDM2-SNP309) has been reported to profoundly accelerate tumor formation, suggesting that it may also represent powerful cancer predisposing allele (Nunobiki et al. 2010). Most studies in different type of cancer found that the G allele increases risk to cancer development (Hu *et al.* 2007; Yang *et al.* 2007; Zhou *et al.* 2007). However, in line with our results, Meissner *et al.* in a study including 72 cervical carcinoma patients and 100 healthy women have shown no significant association between mdm2 SNP309 and cervical cancer (Meissner Rde et al. 2007). This was confirmed in a later study carried out by Hu *et al.* where no significant association was found between MDM2 SNP309 and the study subjects (Hu *et al.* 2010). In addition, several studies found no association between the MDM2 promoter 309 G variant allele and risk to develop various types of cancers.

In a study including 300 cervical cancer patients and 312 cancer-free controls, it was found that G870A polymorphism in CCND1 may not contribute to the etiology of cervical cancer in Chinese and Swedish populations (Jiang et al. 2010). In line with this, in a study carried out by Jeon *et al.* it was found that the CCND1 polymorphism is not associated with an increased risk of squamous cell carcinoma of uterine cervix in Korean women (Jeon *et al.* 2005). However, in a study conducted by Satinder *et al.* , it was revealed that CCND1 (G870A) polymorphism may be associated with increased risk of squamous cell carcinoma (SCC) of the uterine cervix in north Indian women (Satinder *et al.* 2008).

In a pathway-based analysis of genome-wide SNPs (Affymetrix 500k or 5.0 arrays) study including 1076 cases and 1426 controls, several SNPs in the major histocompatibility complex (MHC) were identified as having clear effect on susceptibility to cervical cancer particularly the HLA-DPB1 polymorphism (Ivansson et al. 2011). In addition, the genotype frequencies of the SNPs (the p53 G72C, p21 C31A, CCND1 G870A, and MDM2 promoter T309G) which showed no statistically significant association with cervical cancer were in Hardy-Weinberg equilibrium (at 5% significance level for 1 degree of freedom) (Table 2). Furthermore, testing for deviation from HWE showed that the differences between expected and observed genotypes' counts were not statistically significant as well (Table 2). Therefore, the null hypothesis that the population is in Hardy-Weinberg frequencies is not rejected. Although the widely hold believe that our population has a high level of consanguinity, these results suggest that there is a random mating, no selection, no gene flow and a population large enough to avoid the random effects of genetic drift in our study.

Two prophylactic vaccines are currently available. The quadrivalent vaccine Gardasil protects against HPV types 6, 11, 16 and 18 and was approved by the U.S. Food and Drug Administration in June 2006 for females aged 9 to 26 years. The bivalent vaccine Cervarix protects against HPV types 16 and 18 and was approved by the Australian Therapeutic Goods Administration in April 2007 for use in females aged 9 to 45 years. HPV vaccine is most effective if performed before the onset of sexual activity. Vaccination is still recommended after commencement of sexual activity and even after prior abnormal cytology or CIN, but it is likely to be less effective after HPV exposure.

Improvement in cervical cancer prevention is provided by proper HPV screening and vaccination, which is effective, measures in many infectious diseases. Natural HPV infections are inefficient in eliciting a protective immune response. Vaccines were developed against

HPV infection to prevent cervical cancer and other HPV-related diseases (Harper and Williams 2010). Two types, a bivalent (Cervarix) vaccine that protects against HPV-16 and 18 and a quadrivalent (Gardasil) that is effective against HPV-6, 11, 16 and 18, are being widely introduced in Western countries, and promising new broad-spectrum HPV vaccines are in development. The short term results showed nearly complete efficacy against cervical cytological abnormalities, precancerous lesions, and even genital warts in the case of the quadrivalent vaccine. Our results showed that 70% of cervical tumors are infected with HPV, 56.7% (34/60) are infected with HPV-16 and/or 18 genotypes that are covered by both available vaccines. Therefore, vaccination is expected to protect against more than half of cervical cancers in Saudi Arabia compared to two-third that has been estimated worldwide (Harper 2009). At this time, the duration of efficacy, which is an important determinant of the vaccine, against cervical intraepithelial neoplasia grade 2/3 (CIN 2/3) is 5 years for Gardasil and 8.4 years for Cervarix (Harper and Williams 2010). The long term results will require longer follow-up in order to demonstrate substantial reductions in the rates of cervical cancers particularly in countries without screening programs, in conclusion, 70% of cervical cancer patients were associated with HPV infection. The Linear Array HPV Test Kit has detected 8 different HPV genotypes (16, 18, 31, 33, 45, 52, 59, and 73). The two most common HPV genotypes were 16 and 18 that formed together 80.96% of HPV infections and 56.7% of all cervical cancer patients. These data provide important baseline information for HPV detection and genotyping in cervical cancer that can guide future applications of screening and prevention programs of this disease in the Kingdom of Saudi Arabia.

Furthermore, the p53 G72C, p21 C31A, CCND1 G870A, and MDM2 promoter T309G genetic polymorphic variations were not associated with the occurrence of cervical cancer in Saudi women. These SNPs are not significantly biomarkers utilized for screening individuals who are at high cancer risk to develop cervical cancer. In addition, there were no major differences in the allele frequency or genotype distribution between cervical cancer and controls. The current data reveal negative association between HPV infection and the chosen SNPs in this study. Nonetheless, the paucity in epidemiological data justifies the need to screen women for cervical cancer in the entire Kingdom.

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