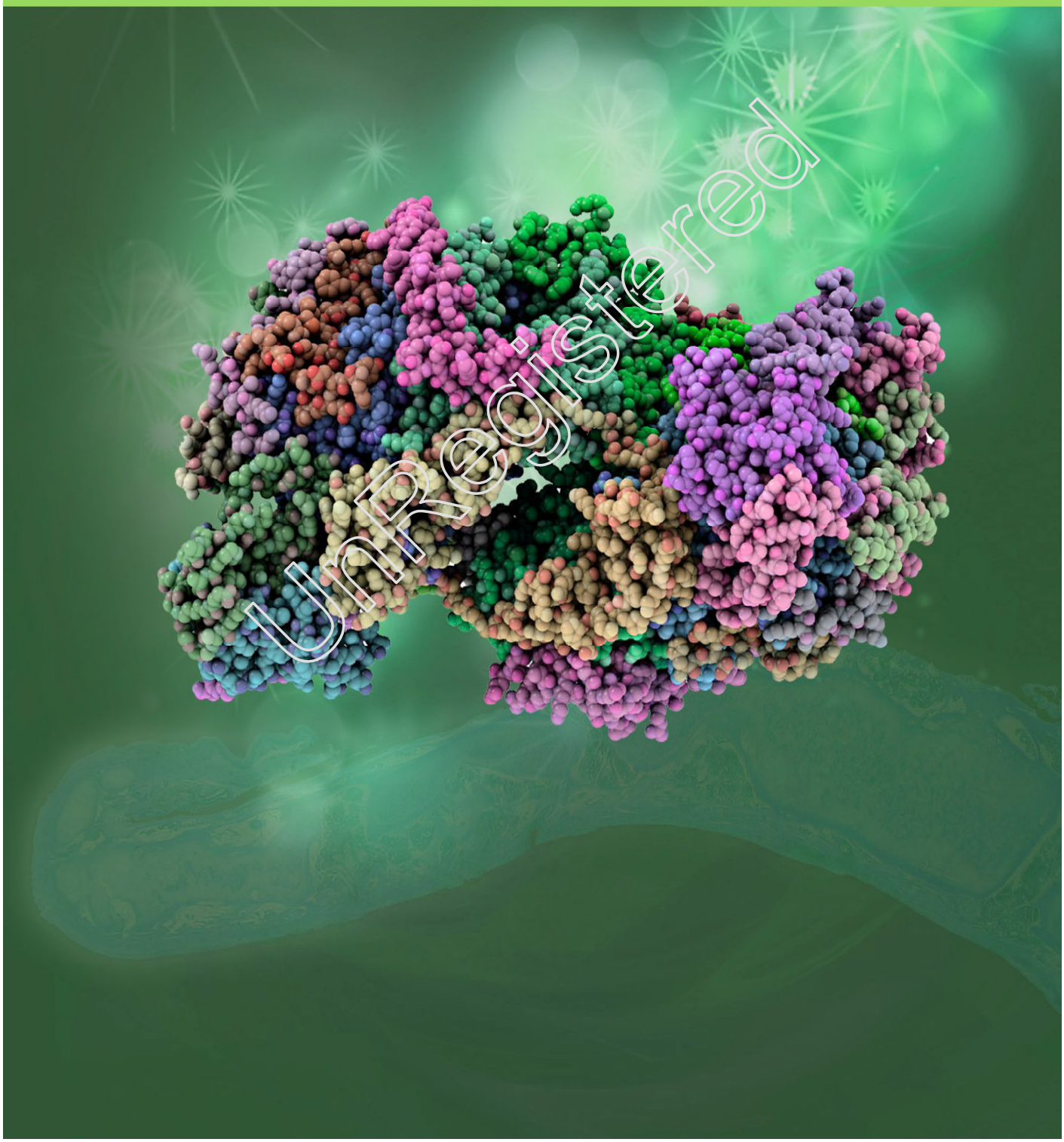
ISSN:2150-1040

Cancer Biology



CANCER BIOLOGY

Editorial Board

2011-2013

CANCER BIOLOGY is a journal that considers full-length articles and Mini Reviews in a broad range of cancer research, from those elucidating advances in understanding the disease processes of cancer to those helping to establish new paradigms in the diagnosis, treatment, and prevention of cancers. Examples of areas covered by Cancer Biology are: Molecular and cellular mechanisms of cancer biology, such as apoptosis, cell cycle/checkpoint regulation, the genetics and epigenetics of tumorigenesis, angiogenesis and lymphangiogenesis, invasion, metastasis, and host immune responses to cancer. Experimental therapeutics for cancers, including the elucidation of mechanisms for the sensitivity and the resistance to current cancer therapies, identification of new therapeutic targets, and development of new cancer therapies.

CANCER BIOLOGY

Monthly Established in October 2010
Volume Number
May10,2011

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The Prognostic Function of Biomarkers in Head and Neck Squamous Cell Carcinomas（HNSCC）

Li-Xia Li[1], Jiang-Xue Wu[1] and Wenlin Huang[1,2]

Abstract: Objective: HNSCC is one of frequently cancers worldwide. Biomarkers would fulfill all three categories: diagnostic, prognostic and therapeutic which are different from other departed biomarkers. These markers could serve as targets for new therapies, which would probably eventually change the outcome of HNSCC. The objective in this review was to highlight recent research about biomarkers that shows prognostic function for HNSCC.

Methods: The data used in this review were obtained mainly from the studies reported in PubMed using the key terms“HNSCC”,“biomarker”and “prognostic”. Original articles and critical reviews selected were relevant to tumor pathogenetic and tumorigenic molecular mechanism.

Results: There are many biomarkers referring to proto-oncogene and tumor suppressor gene, cell cycle regulation, tumor metastasis and immunological markers of virus oncogene. Among them drugs targeting HPV and EGFR have been used for clinic.

Conclusion: Individual inherited diversity is the basis of tumor markers. Because of complicated signal network and reciprocal cross in diverse pathways in most tumors, various kinds of biomarkers should be specifically combined in time of judging tumor prognosis.

Key words: Head and neck cancer squamous cell carcinomas, Biomarker, Prognosis, Evaluation

Introduction

Head and neck cancer is the fifth common cancer worldwide with approximately 650,000 new cases reported annually(1). There are estimated 35,720 new cases of oral, pharynx cancer and 7,600 expected deaths in USA in 2009. More than 90% head and neck cancer are head and neck cancer squamous cell carcinomas, arising from the epithelium lining the sinonasal tract, oral cavity, pharynx, and larynx.

Cigarette smoking, pipes, smokeless tobacco and the use of alcohol are the most important risk factors and their interaction

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may be synergistic in the development of HNSCC. Although significant therapeutic gains have been demonstrated in a number of randomised controlled trials, therapeutic outcome is far from satisfactory, with relatively high loco-regional failure rates of around 50% after 3 years and an overall 5-year survival rate of 50%. The prognosis of HNSCC is influenced by multitude factors about host and tumor.

Improvements in overall survival in patients with HNSCC rest on early identification of pre-malignant lensions and intervention in patients with different methods at risk prior to development of advanced stage disease. Since epithelial carcinogenesis is a multistep process directed by complex molecular events that involved specific genetic defects in proto-oncogene and tumor suppressor gene, early identification of genetic alteration that may represent early transition into malignant phenotype may be possible through various measures recently mentioned.

For the past few years many researches have been carried out about tumor pathogenetic molecular mechanism and abnormal molecular signal chain. A large number of tumor biomarkers emerged after the invention of monoclonal antibody technique. Tumor biomarkers are an indicator of normal biological processes, pathological processes or pharmacologic responding to a therapeutic intervention which can be detected and evaluated objectively[(2)]. A biomarker for HNSCC can be derived from several sources but falls into three general sources: primary tissue, surgical margin tissue, and bodily fluid markers. Three basic types of molecules can be extracted from these sources: DNA, RNA, and protein. Tumor biomarkers are synthesized, secreted by tumors or delivered by host responding to tumors. Biomarkers would fulfill all three categories: diagnostic, prognostic and therapeutic which are different from other departed biomarkers such as CEA, AFP and PSA in sensitivity and specificity. These markers could serve as targets for new therapies, which would eventually change the outcome of HNSCC(Table 1). This article will review the biomarkers' prognostic functions.

Table 1 Potential prognostic markers in HNSCC

Member		Major classes	Common mutation or gene expression	Correlation with
Other growth factors and HNSCC related gene	Cyclin D1	Cell-cycle regulation	Over expression	Worse prognosis
	EGFR	Signal transduction	Over expression	Poor prognosis
	VEGF	Signal transduction	Over expression	Shorter progression-free survival and overall survival and higher recurrence rate
	MMP	Extracellular matrix degradation	Over expression	Lymph node metastasis and poor overall survival
Oncogen	EBV DNA	duplicating of EBV genome lead to malignant transformation of host cell	Elevated loading	Predicting for metastasis
	E6,E7		HPV positive	Better prognosis
Tumour suppressor	P16	Cell-cycle regulation	Loss of expression	Decreased survival

gene	Rb	Cell-cycle regulation	Deletion, methylations, inactivation	Tumor developing
	P53	Cell-cycle regulation	Missense mutation, nonsense mutation, generearrangement, allelic loss and germinal mutation	Poor prognosis
	PTEN	Cell-cycle regulation	Deletion and methylation	shorter overall survival and event-free survival

Proto-oncogene and Tumor Suppressor Gene

Cyclin D1

Cyclin D1 is a proto-oncogene located on chromosome 11q13 that modulates a critical step in cell-cycle control progression. In the advanced time of G1, it is combined with many kinds of cyclin-dependent kinase (CDK) which can make cell enter the stage of S faster, and eventually lead to cell multiplication. The over-expression of cyclin D1 has been demonstrated in 17–79% of tumour specimens from patients with HNSCC which has been shown to correlate significantly with tumour cell differentiation, style of growth extension and metastasis. Patients with cyclin D1-positive tumors had a worse prognosis compared with those with cyclin D1-negative tumors[3]. However some researchers found no significant associations between levels of cyclin D1 and survival of patients with HNSCC[4].

p16

As a tumour suppressor gene p16 is located on chromosome 9p21. P16 was important CDK inactivating protein in the cell cycle regulation having multiple suppressing cancer functions. Some genetic alterations such as loss of p16 expression, mostly homozygous deletions and methylations have been considered to associate with decreased survival[5]. Patients with p16 positive oropharyngeal squamous cell carcinoma (OPSCC) exhibited a significantly better overall survival than those with p16 negative tumors. The improved prognosis of p16 positive OPSCC was found after radiotherapy and surgery[6]. However, downregulation of p16 found in 48% of tumours of HNSCC was associated with a more locally advanced tumour and had no prognostic significance for nodal metastasis and survival[7].

Significant correlation with poor clinical outcome measures of recurrence, metastasis and survival was seen when cyclin D1 amplification and loss of expression of p16 gene occured together than either alone[8].

Retinoblastoma （Rb）

Rb is one of the important tumour suppressor genes located on chromosome 13p14.2. Rb can regulate cell-cycle progression and apoptosis. Rb, i.e. retinoblastoma gene, is a predisposing gene of retinoblastoma. After making analysis of genetics foundation of puerile retinoblastomas, Knudsone verified that Rb gene mutation is related to the genesis of retinoblastomas[9]. Rb gene is the tumour suppressor gene first cloned and completely sequenced. When tumor is developing, the main mutation styles include deletion, mutation, methylations, loss of expression and functional inactivation owing to the combination of virus and oncoprotein. Deletion of Rb is premise of cell multiplication. Nineteen biomarkers were evaluated for protein expression in HNSCC after concurrent cisplatin-based chemoradiation. The result demonstrated that Rb remained significant independent predictive markers for local control. Patients treated with concurrent chemoradiation with high Rb expression had an increased probability of having a recurrence[10].

Cell Cycle Regulation

P53

P53 is located on chromosome 17p13. The protein of p53 is TP53. P53 is a tumor suppressor gene found first which has high associativity with tumor. In 1979 p53 was separated by Lane and Crawford[11] . Wild p53 is a negative growth factor in cell growth cycle which has important biological function in the procedures of cell cycle regulation, DNA damage reparation, cell differentiation, apoptosis and aging. So it is famed with "cell soldier". The ways of p53 gene mutation include missense mutation, nonsense mutation, generearrangement, allelic loss and germinal mutation. The ways of TP53 functional inactivation have different types. For example, TP53 can be degradated when it binds to E6 of HPV, which is related to HPV dependable tumor such as uterine cervix cancer and oropharyngeal cancer. Malignant transformation of B lymphocyte can take place when TP53 binds to EBNA-5 and BZLE1, which is related to EBV dependable nasopharyngeal carcinoma and lymphoma.

The incidence rate of p53 mutation is 50%-69% in HNSCC. p53 mutation may directly influence DNA binding or interfere with formation of TP53 which easily results in tumor recurrence and poor prognosis[12]. Over-expression of mutated p53 protein is associated with tumorous recurrence, poor disease-free survival rate and decreased disease-free survival in HNSCC[13]. A strong correlation was observed between p53 expression in the primary tumor and in the matched lymphnode metastases. The role of p53 in the lymph node metastases was an independent predict or of regional failure and a poor prognosis in patients with HNSCC[14]. Other studies have shown that TP53 mutation or over-expression does not independently predict clinical outcome in patients with HNSCC[15].

For the past few years, recombinant p53 adenovirus (Ad-p53) in combination with chemoradiotherapy has shown very effective in head and neck tumors. In China Ad-p53 gene therapy has been classified to tumour clinical therapeutic drugs[16]. MiR-34[17] and miR-29[18] are indispensable important molecules in p53 gene regulation network which may play a tumor suppressor gene or oncogene role in tumorigenesis. Artificial constructing pre-microRNA has become hot spot of gene regulation research.

PTEN

PTEN is located on 10q23.3. PTEN (MMAC1) was the first tumor suppressor gene found and named by Steck in 1997 which having phosphatase activity. The functions of PTEN mainly include regulating cell cycle, inducing tumor apoptosis and inhibiting tumor cell growth, invasion and metastasis. The ways of PTEN inactivation includes mutation, deletion and methylation, among which the main way is mutation. Somatic mutation or deletion of PTEN has been reported in a variety of tumor types connecting to tumorigenesis. Genetic analysis of PTEN in HNSCC has demonstrated alterations in PTEN, suggesting that PTEN may play a role in HNSCC tumorigenesis. Patients with tongue cancer lacked PTEN expression had a significantly shorter overall survival time and event-free survival time. Lack of PTEN expression may be an independent prognostic indicator for clinical outcome in tongue cancer[19]. PTEN expression in OSCC was related to malignancy grade. Aggressive tumors with a high score of malignancy did not express PTEN, and the PTEN expression was present in the epithelium adjacent to the tumor [20].

EGFR

The EGFR family includes EGFR (c-erbB1 or Her1), c-erbB2 (Her2-neu), c-erbB3 (Her3) and c-erbB4(Her4). They are all

transmembrane protein which not only has receptor's function but also directly transforms extracellular signal to intra-cellular effect. So EGFR family is an important regulatory factor of cell growth, differentiation and survival. The abnormality of signal transduction mediated by EGFR family has significant relation with tumorigenesis and tumor development.

The state of EGFR has been associated with prognosis of HNSCC. Mutations of EGFR are often located on tyrosine kinase. Patients with increased EGFR copy number had a worse median time to progression (TTP) and time to death[21]. Patients without EGFR mutations whose tumor samples showed phosphorylated EGFR had poor prognosis in HNSCC who had not been treated with EGFR molecular targeting therapy[22]. In HNSCC 80%~100% of tumors have increased EGFR protein level. EGFR over-expression correlates with a poor prognosis and decreased overall survival[23]. However several studies have found no association between EGFR and clinical stage, recurrence or survival[24].

The application of target drug began with Dr. Stanley Cohen's stating about EGF in 1960. Tyrosine kinase inhibitor (TKI) (such as Gefitinib and Erlotinib) and EGFR antibody（Cetuximab）have been widely used in advanced non small cell lung cancer, colon carcinoma and head and neck cancer clinically.

Tumor Metastasis

VEGF

VEGF is an important factor in angiogenesis. VEGF induces proliferation, migration and survival of endothelial cells during tumorous growth by binding to specific tyrosine kinase receptor. VEGF has important function in tumor growth and metastasis. There are six members of the VEGF family including VEGF-A~VEGF-E and placenta growth factor. The receptors of VEGF contain VEGFR-1, VEGFR-2 and VEGFR-3. These receptors are all transmembrane protein which over-expression can result in pathological angiogenesis or lymph vessel production. VEGF-A high expression in HNSCC was correlated to shorter progression-free survival and overall survival and higher recurrence rate[25]. Over-expression of VEGF-A and VEGF-C have been correlated to poor overall survival in patients with advanced stage HNSCC[26]. However some studies showed no correlation between VEGF levels and overall survival in native tumor tissue. The results might be attributed to the tumor stroma containing additional VEGF-producing normal cells[27].

The anti–VEGF receptor antibody, bevacizumab, have been approved by the US Food and Drug Administration for first and second line treatment in metastatic colorectal cancer, first-line treatment in advanced non small cell lung cancer, advanced or metastatic renal cell carcinoma and Her-2-positive advanced breast cancer. But further clinical trial should be done to verify bevacizumab's function in HNSCC.

MMP

MMP family plays an important role in tumor invasion and metastasis. MMP is a family of zinc-dependent proteolytic enzymes that degrade most components of the extracellular matrix including collagen, elastin, fibronectin and gelatin which are very important in tumorous invasion and metastasis in HNSCC. The over-expression MMP members in HNSCC contain MMP-2, MMP-7, MMP-8, MMP-9, and MMP-13. Among them MMP-2 and MMP-9 are believed to play major roles. The high expression of MMP-2 in HNSCC was related to advanced tumor stage, lymph node metastasis and bad grade[28]. MMP-9 was over-expressed in 60%-92% of HNSCC which was correlated with poor overall

survival[29]. However, there are also some contrary results[30]. Over-expressed MMP-7[31]and MMP-13[32] were related to poor survival.

Sometimes the results of researches about biomarkers are inconsistent. The discordance between these researches may be due to the difference in the standard of detection method, cases selection and the variation of the tissue handling and analysis technique. The reliability of biomarkers must be validated by random clinical test in the last.

Immunological Markers of Virus Oncogene

Epstein Barr Virus (EBV)

EBV is etiological factor of most Nasopharyngeal carcinoma (NPC). In respect of epidemiology, treatment, and potential biomarkers, NPC has distinct entity compared to other HNSCCs. The role of EBV in the pathogenesis of this disease, particularly in endemic populations, makes this virus an attractive candidate as a clinically useful biomarker. Not only the EBV genome but also many kinds of EBV specific antigens are present in the NPC cells. Quantitative analyses of anti-EBV serology and EBV DNA have become means in early detection, disease surveillance and evaluating prognosis. EBV DNA can be detected in 95% of NPC. Plasma EBV DNA load is an independent prognostic factor, especially in predicting for metastasis. Plasma EBV DNA showed more superior effects in prognosis compared to other clinical parameters in NPC patients receiving concurrent chemoradiotherapy[33]. The Epstein-Barr Nuclear Antigen gene-1 (EBNA1) can be detected before tumorigenesis which is one of early antigens appearing in patient after EBV infection. It is indispensable to latent infection of EBV, keeping and duplicating of EBV genome. Now it is widely used clinically to detect EBNA1 antigen-antibody which is looked as serological index of NPC[34].

The Human Papilloma Virus (HPV)

HPV has been accepted as the causative agent in human cervical cancer. There are over 110 different genotypes of HPVs among which genotypes 16 and 18 are considered as “high risk” HPVs. Two main viral oncogene products, E6 and E7, is related to cell transformation and oncogenicity. After E6 and E7 bind to and inactivate the tumor suppressor gene p53 and pRb, respectively, they lead to malignant transformation of host cell.

HPV is also a causative agent in oropharyngeal cell carcinomas. Approximately 20% of HNSCC are HPV positive and about half of the oropharyngeal cell carcinomas are positive for HPV detection. HPV positive tumors demonstrate the loss of Rb and cyclin D1 expression, over-expression of p16, and rarely have p53 mutation. On the other hand, HPV negative tumors have the contradictory characteristic[35]. HPV positive HNSCC population appears to be biologically and clinically different from HPV negative patients. HPV positive patients are seldom likely to be a smoker or addicted to drinking. HPV positive tonsillar tumors have a better prognosis, including a 60%~80% risk reduction of death, than their HPV negative counterparts. This may in part be due to the fact that HPV positive tonsillar tumors tend to occur in non-smokers and non-drinkers, where p53 mutations are uncommon[36]. HPV-positive oropharyngeal cancers also show good outcome[37]. The presence of HPV confers a survival advantage among HNSCC patients, particularly when p53 is wild type[38]. HPV can be detected from not only tumor biopsy but also salivary rinses. Patients with presence of HPV-16 DNA in surveillance salivary rinses are at significant risk for recurrence. Quantitative measurement of salivary HPV-16 DNA has promise for

surveillance and early detection of recurrence[39]. Currently, clinical trials for HPV positive patients by lowering treatment intensity are being carried out in order to minimizing unnecessary toxicities.

Now two types of HPV vaccines, Cervarix and Gardasil, have been used worldwide to prevent cervical cancer. These two vaccines have no therapeutic effect and are not appropriate for patients who have suffered from cervical cancer. Clinical trials will be done to validate these two vaccines' therapeutic effect in other HPV-dependent tumor such as partial HNSCCs.

Conclusions

So some tumor biomarkers can be taken as tools to measure tumorous progress because of their distinct characteristic. Many kinds of molecular passageway relating to tumorigenesis and tumor development have been discovered with the advancement of oncobiology and tumor genetics. Besides frequently used biomarkers reviewed above，the function of telomerase can not be ignored of. Up to now telomerase is the most broad-spectrum tumor biomarkers. Telomerase's activity has been reported to be up-regulated in HNSCC. It can be looked as an independent prognostic factor for survival in HNSCC[40]. There are different phenotypic biomarkers in different tumor types. Individual inherited diversity is the basis of tumor markers. In view of thinking highly of the transformation of translational medicine, there will be more biomarkers to be used for tumorous screening, diagnosis, therapy and prognosis. Target of targeted therapy is essentially tumor biomarkers. For the past few years drugs targeting HPV and EGFR have been used for clinic. However the effectivity of targeted therapy is always between 20% and 50% maybe because there are complicated signal network and reciprocal cross in diverse pathways in most tumors. So developing multiple targets therapeutic alliance seems very important. In addition, biomarkers are different in tumorigenic various stages. For example, prognostic value of colon carcinoma's biomarker has stage specificity. Tumorigenic multiple factor and individual variation decide that various kinds of biomarkers should be specifically combined in time of judging tumor prognosis and predicting curative effect. An assessment scoring system of integrated evaluation should be established to undertake individualized treatment of tumor more objectively.

Acknowledgements

This work was supported in part by the National Basic Research Program of China (973 Program) grant 2010CB 529904.

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Association between genetic polymorphisms of *CYP2A13*, *CYP2A6* and risk of nasopharyngeal carcinoma in southern Chinese population

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Abstract: Background: Cytochrome P450 2A13 (CYP2A13) and 2A6 (CYP2A6) are enzymes expressed in the human respiratory tract, exhibit high efficiency in the metabolic activation of tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). A C→T transition in the *CYP2A13* gene causes Arg257Cys amino acid substitution and a deletion of the *CYP2A6* gene named as *CYP2A6 *4*, both of them result in a significantly reduced activity toward NNK and other substrates. In this case-control study, we investigated the association between the *CYP2A13* and *CYP2A6* variants, smoking status and the risk of developing nasopharyngeal carcinoma (NPC) in the Cantonese population living in southern China.
Materials and Methods: Genotypes of *CYP2A13* and *CYP2A6* genes were analyzed by using polymerase chain reaction-based restriction fragment length polymorphism (PCR-RFLP) assays and two-step PCR method.
Results: Neither the *CYP2A13* -3375*T* variants nor *CYP2A6 *4* variants were associated with risk of NPC (OR = 0.84, 95% CI = 0.59–1.20, and OR = 0.83, 95% CI = 0.58–1.18, respectively) compared with their wild genotypes. Combination analysis showed that individuals with both *CYP2A13 CT* or *TT* variants and *CYP2A6 *4* variants had no association with risk for NPC (OR = 0.71, 95% CI = 0.33–1.52) compared with those with both *CYP2A13 CC* and *CYP2A6 *1/*1* genotypes. No association with the risk of NPC was observed in smokers with *CYP2A13 C/T* polymorphisms or smokers with *CYP2A6 *4* variant polymorphisms (OR = 0.75, 95% CI = 0.43–1.32, and OR = 0.90, 95% CI = 0.27–1.70; respectively), including after stratification of smoking status. Furthermore, we did not observe association between the combination of two gene polymorphisms and smokers and risk of developing NPC, including the stratification of smoking. **Discussions:** Based on the results of this study, the effect of these two CYP2A13 and CYP2A6 enzymes may be not so important in developing of NPC as in other cancers, such as lung cancer.

Key Words: Polymorphism, *CYP2A13*, *CYP2A6*, nasopharyngeal Carcinoma, epidemiology

Introduction

Nasopharyngeal carcinoma (NPC) is rare in most countries and the incidence belows 1/100,000 (Yu and Yuan, 2002). However, it is one of the most common cancers in southern China including Guangdong, Guangxi and Hunan (Yu and Yuan, 2002). The distinct geographical distribution of NPC seems to be related with certain environmental and hereditary factors. Epstein-Barr virus (EBV) is one of the environmental risk factors which is widely recognized to involve in the carcinogenesis of NPC, because of the detection of EBV genomes in NPC tumor cells (Vasef MA, 1997) and elevated serum levels of IgA and IgG antibodies to EBV in NPC patients (Zong YS, 1992). The preserved foods, such as salted fish, plum vegetable and fermented eggs are considered as etiological factors for NPC, since epidemiological studies have

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confirmed high levels of nitrosamine from these foods (Hildesheim, 1993; Yu, 1989). In addition, tobacco smoke is also recognized as consensus risk factor for NPC, through previous studies on the association between smoking and NPC in NPC-endemic areas (Nam, 1992; Vaughan, 1996; Cheng, 1999). Other environmental factors include wood dust, formaldehyde and kitchen smog (Hildesheim, 2001).

Although many environmental factors were associated with NPC, only a few people develop the disease in areas where NPC is endemic, suggesting that genetic difference such as single nucleotide polymorphisms (SNP) may contribute to NPC carcinogenesis. There is accumulating evidence of association between genetic polymorphism in NPC susceptibility. Polymorphisms in human leukocyte antigen (HLA) class Ⅰ and Ⅱ alleles (Hildesheim, 2002), glutathione S-transferase M1 (GSTM1) (Guo, 2008), homozygous for an allele (c2 allele) of the CYP2E1 (Hildesheim, 1997) and X-ray repair cross-complementing group 1 (XRCC1) (Cao, 2006) have been reported with association with NPC susceptibility.

CYP2A13, one of the three members of the human CYP2A enzyme family, is expressed predominantly in the respiratory tract with the highest level observed in the nasal mucosa (Su, 2000). CYP2A6, another member of the CYP2A enzyme family, has also been detected in human nasal mucosa (Su, 1996). Both of these two enzymes are highly active in the metabolic activation of a major tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Su, 2000; Su, 1996). Recently, a 3375C→T variation in exon 5 of *CYP2A13* gene has been identified, and this single nucleotide polymorphism leads to an Arg257Cys amino acid change (Zhang, 2002), and a deletion of the *CYP2A6* gene named as *CYP2A6 *4*, which results in the absence of CYP2A6 protein, has been discovered in a Japanese population (Kamataki, 1999). Functional analysis showed that 3375C→T variation (Zhang, 2002) and deletion of *CYP2A6* (Kamataki, 1999) have significantly reduced their catalytic activity toward NNK and other substrates compared with their wild genotypes. Recently, the relationship between genetic polymorphisms of *CYP2A13* and several cancers, including lung cancer (Wang, 2003; Cauffiez, 2004) and bladder cancer (Song, 2009), have been investigated in several ethnic groups. Polymorphisms of *CYP2A6* also have been studied in lung cancer (Wang, 2003; Miyamoto, 1999) and esophageal cancer (Sepehr, 2004). In this retrospective case-control study, we investigated the association between the *CYP2A13* and *CYP2A6* variants, smoking status and the risk of developing NPC in the Cantonese population living in southern China.

Materials and Methods

Patients and samples

The study group consisted of 437 patients with histopathologically confirmed, untreated NPC and 470 cancer-free controls. The NPC patients were consecutively recruited from February 2001 to September 2003 at Sun Yat-sen University Cancer Center. Disease staging was performed in accordance with the Chinese 1992 TNM staging system (Min, 1994). Population controls were selected from a community screening program for early detection of cancer. For each eligible case, we tried to match one control subject by sex, age (± 5 years) and ethnicity (Cantonese). At recruitment, informed consent was obtained from each subject, and each participant was then interviewed to solicit detailed information on demographic characteristics and lifetime history of tobacco use. Overall, 500 eligible cases and 500 eligible controls agreed to further risk factor interviews administered by a trained nurse-interviewer, with the final study consisting of 437 cases (87.4%) and 470 controls (94.0%) due to lack of information on smoking or inability to collect blood from some subjects. Information was collected on the number of cigarettes smoked per day, the age at which the subjects started smoking, and the age at which ex-smokers stopped smoking. Smokers were considered current smokers if they had smoked up to 1 year before the date of cancer diagnosis (or up to the date of the interview for controls). This study was approved by the Hospital Review Board of Sun Yat-sen

University Cancer Center.

CYP2A13 and *CYP2A6* genotyping

DNA was extracted from peripheral blood cells by using DNA Blood Mini Kit (Qiagen, Hilden, Germany). Genotypes of *CYP2A13* and *CYP2A6* genes were analyzed by using PCR-RFLP assays and two-step PCR method as described previously (Wang, 2003).

To ensure quality control, genotyping was performed with blinding to case/control status, and a 15% masked, random samples of cases (n = 66) and controls (n = 71) was tested twice by different persons, and the results were concordant for all masked duplicate sets.

Statistical analysis

The Hardy-Weinberg Equilibrium was tested to compare the observed and expected genotype frequencies among cases and controls, respectively. χ^2 tests were used to examine the differences in genotype distributions between cases and controls. The associations between *CYP2A13* and *CYP2A6* polymorphisms and risk of NPC were estimated by odd ratios (OR) and their 95% confidence intervals (CI), which were calculated by unconditional logistic regression. Light or heavy smokers were categorized by the 50th percentile pack-year value among controls, *i.e.*, <20 or ≥20 pack-years (cigarettes per day ÷ 20 × years smoked). The ORs were adjusted for age, gender, and smoking status. *P* values < 0.05 were considered as statistically significant. All analyses were performed using the Statistical Analysis System (Version. 6.12, SAS Institute, Cary, NC).

Results

In the present study, 437 NPC cases and 470 controls were recruited. All cases and controls were ethnic Cantonese. Characteristics of age, gender and smoking status of cases and controls are summarized in Table 1.

Allele frequencies and genotype distributions of *CYP2A13* and *CYP2A6* in cases and controls were shown in Table 2. Relatively low frequencies were observed for the minor alleles of both *CYP2A13* 3375*T* and *CYP2A6 *4* alleles in this study, with the former being 0.08 and 0.09, and the latter being 0.08 and 0.10 in cases and controls, respectively. The results were similar to the reports from northern Chinese (Wang, 2003). The distribution of *CYP2A13* 3375*C/T* genotypes among controls (*CC*, 82.8%; *CT*, 17.2%; *TT*, 0%) was consistent with values predicted by the Hardy-Weinberg equilibrium (*P* = 0.92, Chi-square test). The frequencies of these three genotypes among NPC patients (*CC*, 84.9%; *CT*, 14.4%; *TT*, 0.7%) did not differ from the controls (*P* = 0.11, Chi-square test). The distribution of the *CYP2A6 *1/*1*, **1/*4*, and **4/*4* genotypes among controls was 80.7%, 18.7% and 0.6%, respectively and was also in accordance with the Hard-Weinberg equilibrium (*P* = 0.87, Chi-square test). The distribution of these *CYP2A6* genotypes among NPC patients (83.5%, 15.3%, and 1.2%) were not significant different from controls (80.7%, 18.7%, and 0.6%) (*P* = 0.31, Chi-square test). Unconditional logistic regression analysis was used to estimate associations between the genotypes and risk of NPC (Table 2). Since the *CYP2A13 TT* genotype and *CYP2A6 *4/*4* genotype were rare in this study in both cases (0.7% and 1.2%, respectively) and controls (0% and 0.6%, respectively), so the *TT* genotype was combined with *CT* genotype and **4/*4* genotype was combined with **1/*4* genotype for the subsequent estimation of risk for NPC. Neither the *CYP2A13* -3375*T* variants nor *CYP2A6 *4* variants were associated with increased risk of NPC (OR = 0.84, 95% CI = 0.59–1.20, and OR = 0.83, 95% CI = 0.58–1.18, respectively) compared with their wild genotypes. We next investigated whether there was a statistical interaction between the *CYP2A13* and *CYP2A6* genotypes associated with the risk of NPC. Combination analysis showed that individuals with both *CYP2A13 T* variants and *CYP2A6 *4* variants had no association with risk for NPC (OR = 0.71, 95% CI = 0.33–1.52) compared with those with both *CYP2A13 CC* and *CYP2A6 *1/*1* genotypes (Table 3).

Then we investigated whether an interaction existed between the examined genetic polymorphisms and smoking status. No association with the risk of NPC was

observed in *CYP2A13 C/T* polymorphisms in smokers (OR = 0.75, 95% CI = 0.43–1.32), including after stratification of smoking status (Table 4). We did not observe the association between the *CYP2A6 *4* variant polymorphisms in smokers and risk of developing NPC (OR = 0.90, 95% CI = 0.27–1.70), even after stratification of smoking status (Table 5). Furthermore, we investigated the combination of two gene polymorphisms and smoking status. No association was observed between the two gene polymorphisms and smokers and risk of developing NPC, including the stratification of smoking status (Table 6).

Table 1. Characteristics of 437 cases and 470 control subjects

Variable	No. of Cases (%)	No. of Controls (%)	*P* value
Gender			
Male	234 (53.5)	232 (49.3)	0.11
Female	203 (46.5)	238 (50.7)	
Mean age (years)[a]	46.1 (12.1)	45.6 (15.7)	
Smoking status			
Non-smokers	157 (35.9)	321 (68.3)	< 0.001
Smokers[b]	280 (64.1)	149 (31.7)	
< 20 pack-years	97 (34.6)	65 (43.6)	< 0.001
≥ 20 pack-years	183 (65.4)	84 (56.4)	
Mean pack-years[a]	24.7 (15.6)	7.5 (14.7)	
Median pack-years	20	20	

[a] The values in parentheses are standard deviation; [b] Smokers included 4 ex-smokers.

Table 2. Genotypes and allele frequencies of *CYP2A13* and *CYP2A6* genotypes among cases and controls and their association with risk of nasopharyngeal carcinoma

Genotype	No. of Cases (%)	No. of Controls (%)	Adjusted OR[a] (95% CI)	*P* value
CYP2A13				
CC	371 (84.9)	389 (82.8)	1.00	
CT	63 (14.4)	81 (17.2)	0.80 (0.56-1.15)	0.23
TT	3 (0.7)	0 (0)		
CT+TT	66 (15.4)	81 (17.2)	0.84 (0.59-1.20)	0.34
T allele frequency	0.08	0.09		
CYP2A6				
**1/*1*	365 (83.5)	379 (80.7)	1.00	
**1/*4*	67 (15.3)	88 (18.7)	0.80 (0.55-1.14)	0.22
**4/*4*	5 (1.2)	3 (0.6)	1.76 (0.40-7.83)	0.45
**1/*4+*4/*4*	72 (16.5)	91(19.3)	0.83 (0.58-1.18)	0.31
**4* allele frequency	0.08	0.10		

[a] Odds ratios (OR) and 95% confidence intervals (CI) were calculated with the *CYP2A13* -3375*CC* or *CYP2A6 *1/*1* genotypes as the reference group and adjusted for age, sex and smoking status.

Table 3. Combined effect of *CYP2A13* and *CYP2A6* genotypes with risk of nasopharyngeal carcinoma

CYP2A13	*CYP2A6*	No. of Cases	No. of Controls	Adjusted OR[a] (95% CI)	*P* value
CC	**1/*1*	312	315	1.00	
CC	**1/*4+*4/*4*	59	74	0.82 (0.56-1.22)	0.33
CT+TT	**1/*1*	12	17	0.83 (0.55-1.25)	0.37
CT+TT	**1/*4+*4/*4*	13	17	0.71 (0.33-1.52)	0.38

[a] OR and 95% CI were calculated with the *CYP2A13* -3375*CC* and *CYP2A6 *1/*1* genotypes as the reference group and adjusted for age, sex and smoking status.

Table 4. Association between *CYP2A13* genotypes and risk of nasopharyngeal carcinoma stratified by smoking status

CYP2A13	Smoking status	No. of Cases	No. of Controls	Adjusted OR[a] (95% CI)	*P* value
CC	NO	128	265	1.00	
CT+TT	NO	29	56	0.95 (0.57-1.58)	0.85
CC	YES	243	124	1.00	
CT+TT	YES	37	25	0.75 (0.43-1.32)	0.32
CC	pack-years < 20	89	53	1.00	
CT+TT	pack-years < 20	8	12	0.42 (0.16-1.11)	0.08
CC	pack-years ≥ 20	154	71	1.00	
CT+TT	pack-years ≥ 20	29	13	1.01 (0.48-2.08)	0.99

[a] OR and 95% CI were calculated with the *CYP2A13* -3375*CC* genotype as the reference and adjusted for age and sex.

Table 5. Association between *CYP2A6* genotypes and risk of nasopharyngeal carcinoma stratified by smoking status

CYP2A6	Smoking status	No. of Cases	No. of Controls	Adjusted OR[a] (95%CI)	*P* value
**1/*1*	NO	129	258	1.00	
**1/*4+*4/*4*	NO	28	64	0.83 (0.49-1.38)	0.46
**1/*1*	YES	236	124	1.00	
**1/*4+*4/*4*	YES	44	27	0.90 (0.52-1.55)	0.70
**1/*1*	pack-years < 20	84	54	1.00	
**1/*4+*4/*4*	pack-years < 20	13	12	0.68 (0.27-1.70)	0.40
**1/*1*	pack-years ≥ 20	152	70	1.00	
**1/*4+*4/*4*	pack-years ≥ 20	31	15	0.97 (0.48-1.96)	0.94

[a] OR and 95% CI were calculated with the *CYP2A6 *1/*1* genotype as the reference and adjusted for age and sex.

Table 6. Association between *CYP2A13* and *CYP2A6* genotypes and risk of nasopharyngeal carcinoma stratified by smoking status

CYP2A13	*CYP2A6*	Smoking status	No. of Cases	No. of Controls	Adjusted OR[a] (95% CI)	*P* value
CC	**1/*1*	NO	106	212	1.00	
CC	**1/*4+*4/*4*	NO	22	53	0.80 (0.45-1.41)	0.43
CT+TT	**1/*1*	NO	23	45	0.92 (0.52-1.63)	0.77
CT+TT	**1/*4+*4/**	NO	6	11	0.87 (0.28-2.69)	0.81

	4						
CC	**1/*1*	YES	206	103	1.00		
CC	**1/*4+*4/*4*	YES	37	21	0.94	(0.51-1.73)	0.83
CT+TT	**1/*1*	YES	29	19	0.78	(0.41-1.48)	0.44
CT+TT	**1/*4+*4/*4*	YES	7	6	0.62	(0.20-1.91)	0.40
CC	**1/*1*	pack-years < 20	77	43	1.00		
CC	**1/*4+*4/*4*	pack-years < 20	12	10	0.68	(0.25-1.86)	0.45
CT+TT	**1/*1*	pack-years < 20	7	10	0.34	(0.11-1.04)	0.06
CT+TT	**1/*4+*4/*4*	pack-years < 20	1	2	0.20	(0.02-2.42)	0.21
CC	**1/*1*	pack-years ≥ 20	129	60	1.00		
CC	**1/*4+*4/*4*	pack-years ≥ 20	25	11	1.06	(0.47-2.35)	0.89
CT+TT	**1/*1*	pack-years ≥ 20	22	9	1.16	(0.50-2.71)	0.73
CT+TT	**1/*4+*4/*4*	pack-years ≥ 20	6	4	0.83	(0.22-3.07)	0.77

[a] OR and 95% CI were calculated with the *CYP2A13* -3375*CC* and *CYP2A6 *1/*1* genotype as the reference group and adjusted for age and sex.

Discussion

In this study, we examined whether genetic polymorphisms of *CYP2A13* and *CYP2A6*, alone and in combination, could have an impact on risk for developing NPC. Furthermore, we investigated the gene-environmental interactions with the risk of NPC. On the basis of 437 patients with NPC and 470 controls in a Cantonese population, we did not observe the polymorphisms of *CYP2A13* or *CYP2A6* influenced risk of developing NPC, including the combination of *CYP2A1*3 and *CYP2A6*. Furthermore, we did not observe association between risk of NPC and *CYP2A13* and *CYP2A6* variant genotypes, including when smoking was additionally stratified by pack-years smoked.

The expression of CYP2A13 in human tissue was previsously reported predominantly in the respiratory tract (Su, 2000), and also observed in non-cancerous nasopharynx and NPC tissues (Jiang, 2004). CYP2A6 is mainly expressed in the liver, but is also found at low levels in other extra-hepatic organs including nasal tissue (Su, 1996). The expression of CYP2A13 and CYP2A6 in nasopharynx and the reported enzymatic activity relevant to carcinogens metabolism motivated us to investigate the relation between the genetic polymorphisms of *CYP2A13* and *CYP2A6* in NPC patients and evaluate possible association with environmental factors, such as smoking.

The single mutation of 3375C→T in *CYP2A13* was firstly identified by Zhang et al (2002), and then several groups reported the SNP in different tumors, including lung cancer (Wang, 2003; Cauffiez, 2004; Song, 2009). Wang et al (2003) found the variant allele of *CYP2A13* 3375*T* had a significantly reduced risk for the development of tobacco smoking-related lung cancer, furthermore, the protective effect of the SNP depends on smoking dose. Jiang et al (2004) reported no significant association between *CYP2A13* 3375*C/T* polymorphisms and risk of NPC in Cantonese. We performed another independent cohort in NPC patients and Cantonese controls, and furthermore, we included *CYP2A6* variants in this study, with additional smoking status. We did not observe the association between risk of NPC and *CYP2A13* variant genotypes, including when smoking was additionally stratified by pack-years smoked. This shows the function of CYP2A13 enzyme in developing NPC maybe different from lung cancer. The

published studies regarding the association between the *CYP2A6* polymorphisms and lung cancer risk are conflict (Miyamoto, 1999; Tan, 2001). In addition, this defective *CYP2A6* allele showed no effect in a French population (Loriot, 2001). The group from Thailand (Tiwawech, 2006) investigated the relationship between NPC and *CYP2A6* polymorphisms in 74 NPC patients and 137 age-matched healthy controls, by distinguishing between a wild type allele, ***1***, and two mutant alleles, ***1B*** and ***4***. Overall, a significant association between *CYP2A6* polymorphism and NPC development was observed. Individual with mutant alleles had an increased risk for NPC when compared to those with ***1/*1** genotype (OR = 2.37, 95% CI = 1.27–4.46). We enlarged the NPC patients and healthy controls to 437 and 470 and investigate the association between *CYP2A6 **4** variants and risk of NPC. But no association was observed between *CYP2A6 **4** variant and NPC risk when comparing with *CYP2A6 **1/*1** wild genotype, even after additional assessment with stratification by smoking status. The reason for inconsistent results between our study and Thailand's group maybe: 1, the samples from Thailand are small which may results in overestimate of the OR; 2, we focused on ***4*** variants genotypes of *CYP2A6* and Thailand's group investigated the combination of ***1B*** and ***4*** mutant alleles. We think our results maybe reflect the truly relationship between *CYP2A6 **4** polymorphism and risk of NPC.

The polymorphisms of *CYP2A13* and *CYP2A6* will result the reduced enzymatic activity in carcinogen, and results from lung cancer show the enzymes have important function in tumorigenesis of lung cancer (Wang, 2003; Miyamoto, 1999). We did not observe the relationship between polymorphisms of *CYP2A13* and risk of NPC, which was consistent with the results of Jiang et al (2004), neither the association of *CYP2A6 **4** variants and risk of NPC. Based on the results of this study, it shows that the effect of these two CYP2A13 and CYP2A6 enzymes are not so important in developing of NPC as in lung cancer.

In summary, no strong association was observed between the variant alleles of *CYP2A13* and *CYP2A6* and risk of developing NPC in the Cantonese population of southern China. Furthermore, we did not observe the interaction between genetic polymorphisms of *CYP2A13* and *CYP2A6* and smoking status in risk to develop NPC. The effect of these two enzymes maybe not so important in tumorigenesis of NPC as in lung cancer.

Acknowledgements

This work was supported in part by the Chinese State Key Basic Research Project (No. 2011CB504805), National High Technology Research and Development Program of China (863 Program) (No. 20060102A4002), and a grant of 985 Project from Ministry of Education of P. R. China.

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Genotoxic Effects Of Organophosphate Pesticide Phorate In Some Exotic Fishes Of Kashmir

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ABSTRACT: Genotoxic effects of phorate, a commonly used pesticide were evaluated in two exotic sub-species of fish, *Cyprinus carpio* L. (family *Cyprinidae*) namely *Cyprinus carpio specularis* and *Cyprinus carpio communis* using micronucleus test. Genotoxicity of said pesticide was confirmed by incidence of micronucleus in peripheral erythrocytes using three sub-lethal concentrations viz 0.2ppm, 0.4ppm and 0.6ppm of phorate after 24, 48 and 72 hours. All the three concentrations were able to induce micronuclei formation in erythrocytes of both fish species. However, after 48h and72h, a statistically significant increase was found in the frequency of micronuclei in peripheral erythrocytes of both fish species. The percentage of single micronuclei in *Cyprinus carpio specularis* (0.03 ± 0.01 in control) increased to 1.15 ± 0.32 from low to high concentrations after 24h and 2.74 ± 0.52 in longer exposures. In *Cyprinus carpio communis* somewhat similar results were observed with increase in percentage of single micronuclei (0.03 ± 0.01 in control) to 1.30 ± 0.23 at 24h from low to high concentration and this percentage continued to increase by 2.08 ± 0.31 and 2.91 ± 0.39 after 48 and 72 h respectively (Mann-Whitney U test; $p< 0.05$).

Key words: Micronucleus; *Cyprinus*; Phorate; Genotoxicity; Pesticide.

INTRODUCTION

Phorate is an organophosphate pesticide effective against a wide array of insects, mites and some nematodes. It is used on a variety of field crops but 80% of its use is on corn, potatoes and cotton. Other crops include beans, peanuts, sugar beets, sorghum, wheat and soyabean. In Kashmir phorate is used on a variety of crops especially it is used to control woolly apple aphid.

Phorate is very highly toxic to birds and other wild life. It has been responsible for numerous incidents of mortality in birds and fish. Fish provide a relevant model for the evaluation of aquatic genotoxicity *in situ*, as well as the action of polluted effluents, sediments or toxic compounds (Hayashi *et al*., 1998). The advantage of fish as model organism include the fact that fish respond in a manner similar
to mammalian test species to chemicals that induce peroxisome proliferation in hepatocytes and oxidative damage in hepatocytes. The advantages of using fish as model organisms include the ease with which the fish, especially aquarium species, can be held in laboratory and exposed to toxic chemicals. Since fish often respond to

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toxicants in a manner similar to higher vertebrates, they can be used to screen for chemicals that have the potential to cause teratogenic and carcinogenic effects in humans. With the last decade, the use of fish as appropriate models for genetic monitoring of toxic chemicals in aquatic environments has become popular (Pacheco and Santos, 1998).

A variety of *in vitro* and *in vivo* assays with fish are being used as a model system for toxicological, biochemical and developmental studies (Powers, 1989). At the cellular level the micronucleus test on various fish tissues is among the most wide spread assessments of genotoxicity in water (Al-Sabti K and Hardig J, 1990). Various investigations using fish as sentile for screening the clastogenic effects of xenobiotics indicate that these fish represent good experimental models for genotoxicity studies (Matsumoto and Colus, 2000; Porto *et al*., 2005, Pantaleao *et al*., 2006).

The aim of the present study is to investigate the genotoxic effects of a known organophosphate pesticide, phorate on two sub-species of *Cyprinus carpio* fish, *Cyprinus carpio communis* (scale carp) and *Cyprinus carpio specularis* (mirror carp), phenotypically differentiable according to the pattern of scales, when exposed to different concentrations of the said organophosphate pesticide, using micronucleus test.

Micronucleus test can be performed on different cell types like lymphocytes, erythrocytes, fibroblasts, and exfoliated epithelial cells, without extra *in vitro* cultivation step. The micronucleus assay is generally accepted as equivalent to the cytogenetic assay in responsiveness to chemical mutagens. As scoring is less consuming, the assay is preferred for routine screening purposes. Another advantage of the micronucleus assay is that micronucleated erythrocytes persist in the peripheral blood for a relatively longer time, therefore allowing the study of chronic exposure to potential mutagens (Choy *et al., 1985*).

Micronucleus assay originally developed with mammalian species, has been extensively used to test for genotoxic activity of chemicals (Heddle *et al.*, 1983). The micronucleus test in fish has a potential for detecting clastogenic activity. Various studies have shown that the peripheral erythrocytes of fish have a high incidence of micronuclei after exposure to different pollutants under fold and laboratory conditions.

MATERIALS AND METHODS

Experimental animals

Two sub-species of *Cyprinus carpio* L. (family *Cyprinidae*) namely *Cyprinus carpio* specularis and Cyprinus carpio communis were used as experimental animals as they are available throughout the year in Kashmir .Adult specimens of both sub-species were collected from Dal-lake and were identified on the basis of their scales. Fish were acclimatized for 45 days at 28^0C prior to trials (Anitha *et al.,* 2000)[10]. Specimens were kept in polypropylene troughs each with 7-8 individuals/50 L of water. Water was kept O_2 saturated by aeration. The troughs were cleaned daily and the water as well as the pesticide was renewed to keep the concentration constant throughout the test period of 24, 48, 72 h. Control fish were kept in dechlorinated tap water without any treatment. Fish were fed commercial fish feed daily at least one hour prior to the replacement of water. Only healthy, active fish starved for 24hr were used for the experiment and they were allowed no food during treatment procedures.

Insecticide chemical and dose selection

The commercial grade of phorate was obtained from G.M.Shah pesticides, Srinagar; manufactured from Cyanamid, India Ltd. (Bombay).On the basis of literature data (LC_{50} values for phorate), three sub-lethal concentrations (0.2ppm, 0.4ppm and 0.6ppm) of phorate were then selected for the experiment.

Experimental design

Group I (Control): One group of fish from each sub-species was selected as control, kept in chlorinated water without any treatment. They were fed once daily commercial fish feed.

Group II: The fish sample from two sub-species was subdivided into three sub groups each, based on the dose selection of

the pesticide. All subgroups had equal number of fishes (five fish/group/duration) maintained in 50litre/polypropylene troughs. After treatment with pesticide, the frequencies of micronuclei in all experimental sub-groups were examined at three durations (24, 48 and 72h) and at each concentration.

Cytogenetic studies using micronucleus test

The method of Schmid (1975) was used. The fish were injected with 0.1 ml of 0.025% colchicine and sacrificed two hours later with a slight blow on the head region. Chemical treated and control fish were cut in the caudal. From the freshly collected blood, smears were made on grease free slides. After fixation using methanol as fixative, slides were stained with Mayer's hematoxylin, rinsed in Scott's tap water substitute, followed by another staining in eosin (Pascoe and Gatehouse, 1986).After completion of this staining process, the slides were then washed in 30%, 50%, 70% and 90% alcohol, cleaned in xylene and mounted using D.P.X. The slides were then examined using a simple light microscope. For each concentration and duration, five fish specimens were used and from each fish, six slides were studied and 1200cells were scored under 600x magnification.

STATISTICAL ANALYSIS

Statistical analysis of data to verify the significant difference in the incidence of micronuclei between treated and control groups at 0.05 and 0.01 level of significance was performed using non-parametric criteria, Mann-Whitney U test to analyze the frequency of micronuclei.

RESULTS

The genotoxicity of phorate in *Cyprinus carpio specularis* and *Cyprinus carpio communis* was confirmed by incidence of micronucleus in peripheral erythrocytes after 24, 48 and 72 hours. Three sub-lethal concentrations of phorate, 0.2 ppm, 0.4 ppm and 0.6 ppm were used, and it was observed that all these concentrations were able to induce micronucleus formation in erythrocytes of both the fish species. No increased incidence of micronucleated erythrocytes of *Cyprinus carpio specularis* and *Cyprinus carpio communis* was reported with sub lethal concentration of 0.2 ppm after 24-hour intervals. However, after 48h and 72h, a statistically significant increase was found in the frequency of micronuclei in peripheral erythrocytes of both fish species. The percentage of single micronuclei in *Cyprinus carpio specularis* (0.03±0.01 of control) increased to 1.15±0.32 from low to high concentrations after 24 h and continued to increase by 1.78±0.30 and 2.74±0.52 in longer exposures table 1 and table 2).

In *Cyprinus carpio communis* somewhat similar results were observed with increase in percentage of single micronuclei (0.03±0.01 control) to 1.30±0.23 at 24 h from low to high concentration and this percentage continued to increase by 2.08±0.31 and 2.91±0.39 after 48 and 72 h respectively.

Statistical analysis showed significant difference in the frequency of incidence of micronuclei in the erythrocytes of control and frequency of micronuclei in the erythrocytes of phorate treated groups of *Cyprinus carpio specularis* and *Cyprinus carpio communis* respectively (Mann-Whitney *U* test; $P < 0.05$). Both dose and time dependent increase in the micronuclei frequency was observed in treated fish species and a peak value detected at higher concentrations after 72h of phorate injection, clearly showed a higher incidence of micronucleated peripheral erythrocytes.

Table 1: Micronucleus frequencies (%) in peripheral blood erythrocytes of *Cyprinus carpio specularis* exposed to different concentrations of phorate

Treatment	Concentration	MN frequencies (%)		
		24 h	48 h	72 h
		Mean ± SD	Mean ± SD	Mean ± SD

Control	-	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Phorate	0.2 ppm	0.06 ± 0.03	0.09 ± 0.03*	0.23 ± 0.03*
	0.4 ppm	0.34 ± 0.11*	0.54 ± 0.15*	0.88 ± 0.12*
	0.6 ppm	1.15 ± 0.32*	1.78 ± 0.30*	2.74 ± 0.52*

(Mann-Whitney U test) * P < 0.05

Table 2: Micronucleus frequencies (%) in peripheral blood erythrocytes of *Cyprinus carpio communis* exposed to different concentrations of phorate

Treatment	**Concentration**	**MN frequencies (%)**		
		24 h	**48 h**	**72 h**
		Mean ± SD	**Mean ± SD**	**Mean ± SD**
Control	-	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Phorate	0.2 ppm	0.04 ± 0.01	0.08 ± 0.03*	0.28 ± 0.01*
	0.4 ppm	0.39 ± 0.085*	0.67 ± 0.06*	1-05 ± 0.26*
	0.6 ppm	1.29 ± 0.23*	2.08 ± 0.31*	2.91 ± 0.39*

(Mann-Whitney U test) * P < 0.05

DISCUSSION

Organophosphate insecticides are ubiquitous environmental contaminants because of their wide applications in agriculture. It is known that in fishes organophosphate pesticides are neurotoxic and they inhibit acetylcholinesterase activity with subsequent disruption of nervous functions, thereby interfering with some of the vital physiological functions (Rao and Rao, 1983). Since organophosphate pesticides are finding increasing use in recent years, this can result in acute and long term side effects, including sickness and death of people, useful animals, fish, birds and destruction of crops.

In the present study, positive genotoxic effects, measured as micronucleus frequency in erythrocytes from both fish species (*Cyprinus carpio specularis* and *Cyprinus carpio communis*) exposed to different doses of phorate were observed. The result of the present study revealed a significant induction of micronuclei in peripheral erythrocytes ($p<0.01$ and $p<0.05$) of both fish species. The appearance of inter-specific differences observed in the present study could be attributed to the specificity of DNA repair, cell turnover time, physiological peculiarities, contaminant uptake or biotransformation in the fish species studied.

On the other hand age, sex, reproductive status, genetic constitution may affect micronucleus frequency in fish (Al-Sabti *et al.,* 1994). However, *Cyprinus carpio specularis* and *Cyprinus carpio communis* in the present study were selected from the same age groups. The exposure was performed in the laboratory under standard experimental conditions, Therefore, the inter-specific differences in micronuclei incidences should not be attributed to the intrinsic problems of the experimental system used.

In the present study a significant difference in the micronucleus incidence among treated and control groups was observed. The peak frequency of micronucleated erythrocytes was observed at 72h after exposure.

The length of cell cycle critical to micronuclei formation depends upon the time needed to replicate DNA and perform nuclear division. In man and mice the duration of the cell cycle has been well documented. There is, however, little information on the duration of the cell cycle in the tissues of teleost species since the cell cycle varies with temperature in piokilotherms (Al-Sabti and Metcalfe, 1995). The incubation times were chosen without the benefit of specific knowledge of the times required for the division of fish cells (Al-Sabti, 1994). A time dependent increase in the incidence of micronuclei in peripheral

erythrocytes of *Cyprinus carpio specularis* and *Cyprinus carpio communis* was established and confirms other observations (Al-Sabti, 1986; Nepomuceno and Spano, 1995; Nepomuceno *et al.,* 1997; Gustavino *et al.,* 2001; Buschini *et al.,* 2004). Increased chromosome aberrations and micronuclei were observed in bone marrow cells of rats that received (Dhingra *et al.,* 1990). Phorate in the present study was also found to induce micronuclei in peripheral erythrocytes of fish. The carbohydrate metabolism was found to be adversely affected by phorate in the serum of fresh water fish *Clarius batrachus* (Jyothi and Narayan, 1999). Decreased total serum levels and plasma ChE activity was observed in beagle dogs treated with phorate (Piccirillo *et al.,* 1987).

The present study reveals that micronucleus assay has a great potential for detecting clastogenic substances in aqueous media. However, additional experimental evidence is needed to evaluate these hypotheses. Also further studies on aquatic organisms exposed in-vivo as well as in-vitro to various chemicals is certainly needed to clarify the mechanism of micronucleus formation.

Acknowledgement

The authors are thankful to CSIR New Delhi for providing JRF to Farooq Ahmad Ganai.

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GC-MS Study on the Bioactive Components and Anti-Cancer Activities of *Solanum surattense*

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Abstract: Ayurveda is a 5000 year-old system of natural healing that has its origins in the Vedic culture of India. In the last few decades there has been an exponential growth in the field of herbal medicine. Medicinal plants and herbs contain substances known to modern and ancient civilizations for their healing properties. They were the sole source of active principles capable of curing man's ailments. Thus natural products have been a major source of drugs for centuries. *Solanum surattense*, is such a medicinally important plant of family Solanaceae. All parts of the tree have medicinal properties. Taking into consideration the medicinal importance of the plant, the volatile organic matter from the bark of this plant was analyzed for the first time using GC-MS and the structures were confirmed by genesis. The majority of prevailing constitutents in this plant, trans-Squalene (31.55%), 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (10.20%), Phytol (8.17%) and Vitamin E (7.86%) are proven anti-Cancer agents.

Key words: *Solanum surattense*, GC-MS, Bioactive components, Biological Activity

Introduction

Plants have been an important source of medicine for thousands of years. The rich resource is decreasing at an alarming rate as a result of over-exploitation. The medicinal value of drug plants is due to the presence of some chemical substances in the plant tissues which produce a definite physiological action on the human body. These chemicals include alkaloids, flavanoids, glucosides, tannins, gums, resins, essential oils, fatty oils, carbon compounds, hydrogen, oxygen, nitrogen salts of some chemicals, etc. Very few of these chemicals are toxic also. Hence, preparation and administration of drugs should be done by experts only. Drugs may be obtained from various parts of the plant. So, an extensive study is required to detect the medical properties of the plant. Several medicinal plants have been tried against pathogenic microorganisms [1]

Solanaceae is a large plant family containing two thousand and three hundred species, nearly half of which belong to a single genus, Solanum. There are herbs, shrubs or small trees under this genus. This family comprises a number of plants widely known for the presence of variety of natural products of medicinal significance. Crude plant extract is beneficial in bronchial asthma and non-specific cough, influenza, difficult urination, bladder stones, rheumatism, etc.

Medicinal plants are of great importance to the health of individuals and communities. The extensive use of natural plant as primary health remedies due to their pharmacological properties is quite common [2]. Plants are used medicinally in different countries and are a source of many potent

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and powerful drugs [3].The investigation of the efficacy of plant-based drugs has been paid great attention because of their few side effects, cheap and easy availability [4].According to the world health organization 80% of the world population still relies mainly on plants drugs. Resistance to antibiotics has been the reason of research for newer drugs to treat microbial infections [5].The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, and phenolic compounds [6]. *Solanum surattense* belongs to family Solanaceae. It is a commonly growing perennial herbaceous weed. It is commonly known as Indian night shade or yellow berried night shade has been used traditionally for curing various ailments such as fever, cough, asthma and diabetes in South Indian traditional medicines [7]. The anti diabetic potential of the fruit was studied in diabetic rats [8,9]. The ethanol and methanol extracts of *Solanum surattense* showed strong antibacterial activity against *Pseudomonas aeruginosa* [10]. The present studies were carried out to screen the phytochemical constituents and to test the antifungal efficacy of the seed extracts of *Solanum surattense* with reference to fungal spp. *Solanum surattense* have also proved to be showing antibacterial [11] and antifungal activities. [12]

Materials and Methods

Plant material and extraction procedure

Leaves of *Solanum surattense* were bought fresh from local market, Thanjavur. 10gm powdered plant material was soaked in 20ml of Absolute alcohol overnight and then filtered through a Whatman® No. 41 filter paper (pore size 20 - 25_m) along with 2gm Sodium sulfate to remove the sediments and traces of water in the filtrate. Before filtering, the filter paper along with sodium sulphate was wetted with absolute alcohol. The filtrate is then concentrated by bubbling nitrogen gas into the solution and was concentrated to 1ml. The extract contains both polar and non-polar phytocomponents.

Gas Chromatography–Mass Spectrometry (GC/MS) analysis

GC/MS analysis of this extract was performed using a Perkin Elmer GC Claurus 500 system and Gas Chromatograph interfaced to a Mass Spectrometer (GC/MS) equipped with a Elite-1 fused silica capillary column (30 m × 0.25 mm ID. ×1 µMdf, composed of 100% Dimethyl poly siloxane). For GC/MS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/min. and an injection volume of 2 µl was employed (split ratio of 10:1). Injector temperature 250°C; Ion-source temperature 280°C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10°C/min, to 200°C, then 5°C/min to 280°C, ending with a 9 min. isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 36 min.The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adopted to handle mass spectra and chromatograms was a TurboMass Ver5.2.0

Results and Discussion

Identification of Components

Interpretation on mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The Name, Molecular weight and Structure of the components of

the test materials were ascertained.

Seventeen compounds were identified in *Solanum surattese* leaf extract by GC-MS analysis .The active principles with their Retention time (RT), Molecular formula, Molecular weight (MW) and Concentration (%) are presented in (Table 1 and Fig 1).The prevailing compounds were trans-Squalene (31.55%), n-Hexadecanoic acid (13.30%) and 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- (10.20%).

The biological activities listed are based on Dr. Duke's Phytochemical and Ethnobotanical Databases by Dr. Jim Duke of the Agricultural Research Service/USDA.

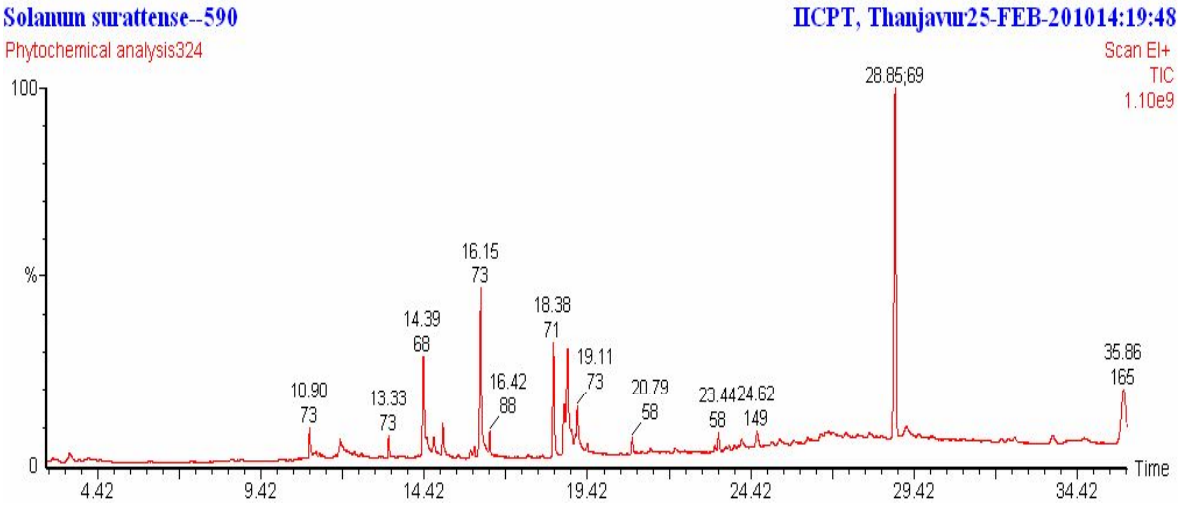

Figure 1: Chromatogram obtained from the GC-MS study with the extract of *Solanum surattense*

Table 1. Total ionic chromatogram (GC–MS) for the ethanol extract of *Solanum surattense* obtained with 70 eV Elite-1 fused silica capillary column with He. gas as the carrier.

No.	RT	Name of the compound	Molecular Formula	MW	Peak Area %
1	3.54	Hexanoic acid, ethyl ester	$C_8H_{16}O_2$	144	1.60
2	10.90	Dodecanoic acid	$C_{12}H_{24}O_2$	200	2.21
3	11.83	(1R,3R,4R,5R)-(-)-Quinic acid	$C_7H_{12}O_6$	192	3.29
4	13.33	Tetradecanoic acid	$C_{14}H_{28}O_2$	228	1.35
5	14.39	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	296	8.54
6	16.15	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256	13.30
7	16.42	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284	1.01
8	18.38	Phytol	$C_{20}H_{40}O$	296	8.17

9	18.71	9,12-Octadecadienoic acid (Z,Z)-	$C_{18}H_{32}O_2$	280	3.54
10	18.81	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	$C_{18}H_{30}O_2$	278	10.20
11	19.11	Octadecanoic acid	$C_{18}H_{36}O_2$	284	2.89
12	19.41	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	312	0.44
13	20.79	2-Propenamide, N-[2-(dimethylamino)ethyl]-	$C_7H_{14}N_2O$	142	1.07
14	24.15	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester [Synonyms: Palmitin, 2-mono-]	$C_{19}H_{38}O_4$	330	1.25
15	24.62	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390	1.73
16	28.85	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- [Synonyms: trans-Squalene]	$C_{30}H_{50}$	410	31.55
17	35.86	Vitamin E	$C_{29}H_{50}O_2$	430	7.86

Table 2: Major Phyto-components and its biological activities obtained through the GC-MS Study of *Solanum surattense* has been listed along with its active biological activities

Sl. No.	**Retention Time**	**Peak Area %**	**Name of the Compound**	**Active biological activity**
1.	3.54	1.60	Hexanoic acid, ethyl ester	Acidulant, Flavor
2.	10.90	2.21	Dodecanoic acid	Flavor
3.	11.83	3.29	(1R,3R,4R,5R)-(-)-Quinic acid	Choleretic
4.	13.33	1.35	Tetradecanoic acid	Flavor, Nematicide and Pesticide
5.	14.39	8.54	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Flavor
6.	16.15	13.30	n-Hexadecanoic acid	Antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor
7.	16.42	1.01	Hexadecanoic acid, ethyl ester	Antioxidant, hypocholesterolemic nematicide, pesticide, anti-androgenic flavor, hemolytic, 5-Alpha reductase inhibitor
8.	18.38	8.17	Phytol	Cancer-Preventive
9.	18.81	10.20	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	Antiinflammatory, hypocholesterolemic cancer preventive, hepatoprotective,

				nematicide, insectifuge, antihistaminic antieczemic, antiacne, 5-Alpha reductase inhibitor, antiandrogenic, antiarthritic, anticoronary, insectifuge
10.	19.11	2.89	Octadecanoic acid	5-Alpha-Reductase-Inhibitor, Cosmetic, Flavor, Hypocholesterolemic, Lubricant,Perfumery, Propecic and Suppository
11.	24.15	1.25	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester [Synonyms: Palmitin, 2-mono-]	Abortifacient, Adrenocorticotrophic, Analgesic, Antialzheimeran, Antiarrhythmic, Antibacterial, Anticholinesterase, Antiinfarctal, Antiinflammatory, Antimalarial, Antipyretic, CNS-Depressant, Hypotensive, Inotropic, Pesticide, Respiradepressant and Uterotonic
12.	24.62	1.73	1,2-Benzenedicarboxylic acid, diisooctyl ester	Insecticide, Larvicide and Pesticide
13.	28.85	31.55	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)- [Synonyms: trans-Squalene]	Antibacterial, Antioxidant, Antitumor, Cancer-Preventive, Chemopreventive, Immunostimulant, Lipoxygenase-Inhibitor, Perfumery, Pesticide and Sunscreen
14.	35.86	7.86	Vitamin E	5-HETE-Inhibitor, Allergenic, Analgesic, AntiMD, AntiMS, AntiPMS, Antiaggregant, Antiaging, Antialzheimeran, Antianginal, Antiarteriosclerotic, Antiatherosclerotic, Antibronchitic, Anticariogenic, Anticataract, Antichorea,

				Anticoronary, Antidecubitic, Antidermatitic, Antidiabetic, Antidysmenorrheic, Antiepitheleomic, Antifibrositic, Antiherpetic, Antiinflammatory, Antiischaemic, Antileukemic, Antileukotriene, Antilithic, Antilupus, Antimastalgic, Antimyoclonic, Antineuritic, Antinitrosaminic, Antiophthalmic, Antiosteoarthritic, Antioxidant, Antiparkinsonian, Antiproliferant, Antiradicular, Antiretinopathic, Antisenility, Antisickling, Antispasmodic, Antisterility, Antistroke, Antisunburn, Antisyndrome-X, Antithalassemic, Antithrombotic, Antithromboxane-B2, Antitoxemic, Antitumor, Antitumor (Breast), Antitumor (Colorectal), Antitumor (Prostate), Antiulcerogenic, Apoptotic, Cancer-Preventive, Cerebroprotective, Circulatory-Stimulant, Hepatoprotective, Hypocholesterolemic, Hypoglycemic, Immunostimulant, Insulin-Sparing, Lipoxygenase-Inhibitor, Ornithine-Decarboxylase-Inhibitor, P21-Inducer, Phospholipase-A2-Inhibitor,

				Protein-Kinase-C-Inhibitor, Protein-Kinase-C-Inhibitor and Vasodilator

Acknowledgement

I thank Sh. S. Kumaravel, Scientist, Department of Food Quality and Testing, Indian Institute of Crop Processing Technology and Dr. K. Alagusundaram, Director, Indian Institute of Crop Processing Technology wholeheartedly for guiding and providing me valuable suggestions, throughout the study. I wish to thank my father Mr. N. Ramanathan, my mother Mrs. R. Latha and my beloved sister, Ms. R. Jamuna, for their contiouous support and encouragement.

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Conservative Breast Surgery In Early And Locally Advanced Breast Cancer

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Abstract: Aim: to evaluate efficacy of breast conservation surgery in loco-regional control of early & locally advanced breast surgery.

Methods: the study included 2 groups; group A: 30 patients with early breast cancer & group B: 32 patients with 33 locally advanced breast cancer which were furtherly subdivided into 2 subgroups: 1-FAC group: 24 patients with 25 breast cancer received 3 cycles of FAC regimen, 2-TAC group: 8 patients received 3 cycles of TAC regimen. Group A patients were submitted to quadrentectomy & axillary evacuation, group B patients were submitted to quadrentectomy & axillary evacuation or modified radical mastectomy according to their response to neoadjuvant chemotherapy.

Results: In group A, 1 patient developed local recurrence & submitted to completion mastectomy, in group B, overall response to neoadjuvant chemotherapy was 54.5%. 14 patients in group B underwent breast conservation surgery, 18 patients underwent modified radical mastectomy, 5 patients in group B developed treatment failure. **Conclusion:** breast conservation surgery is safe surgical technique for local control of both early & locally advanced breast cancer after downstaging by neoadjuvant chemotherapy. Neoadjuvant chemotherapy has significant anti-tumour activity & it increases the ability to perform breast conservation surgery.

Keywords: Breast conservation surgery, neoadjuvant chemotherapy.

Introduction

Randomized controlled trials over the past two decades have now established that mastectomy and breast conserving surgery are equivalent in terms of survival (veronesi et al, 2002).Breast conservation surgery can be used also for treatment of locally advanced breast cancer after downstaging by the use of neoadjuvant chemotherapy. Neoadjuvant chemotherapy for breast cancer was initially used during the seventies of the last century in locally advanced or inoperable disease in order to achieve surgical resection. It was then extended to operable breast cancer with a view to downstaging tumours to facilitate breast conserving surgery. Increasingly, it is being considered as a treatment for earlier-disease stage (Charfare et al, 2005). Neoadjuvant chemotherapy serves as an in vivo sensitivity test, it decreases the incidence of growth spurt at the site of micrometastasis after primary tumour resection & it facilitates the study of cancer biology with the same overall survival & recurrence-free survival rates as the adjuvant

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chemotherapy (Ikeda et al , 2002).Although the chemotherapy regimens have varied widely among studies, most clinical trials have used anthracycline-based regimens e.g. a combination of 5-fluorouracil, doxorubicin & cyclophosphamide (FAC). These regimens are generally prefered because of the higher response rate observed in the metastatic setting (Esteva & Hortobagyi, 1999). The appearance of taxanes has stimulated new excitement in this field, not only because of their high level of activity against metastatic breast cancer as single agents, but also because of their lack of cross-resistance with other drugs, including anthracyclines (Goble & Bear, 2003).

Patients & Methods:

This prospective study included two groups:

Group A : included 30 patients with early breast cancer (T1,2 N0, M0, or T0,1,2 N1, M0)

Group B : included 32 female patients with 33 locally advanced breast cancer (one patient was presented with bilateral disease) (stage IIB: T3 N0 M0, stage IIIA: T0,1,2 N2 M0 & T3 N1,2 M0, & stage IIIB: T4 N0,1,2 M0, or stage IIIC: any T N3 M0) admitted into The Oncology Unit, General Surgery Department, and Clinical Oncology Department, Tanta University Hospital. All the patients were informed by this study & written consents were taken from cases underwent surgery.

Eligible patients had to meet the following criteria: (a) at least 18 years of age (b) satisfactory liver and renal function, (c) life expectancy ≥9 months, (d) WHO performance score of 0–1 and ability to understand medical advice, and (e) full clinical-pathological examination and good staging for the patients including mammography, mammosonography, tissue diagnosis that was obtained by FNAC or open biopsy, & evaluation of the cardiac status by ECG & echo-cardiogram done for all the patients before the start of the anthracycline-based chemotherapy regimens, after the third cycle , and at the end of chemotherapy to detect any cardiac toxicity.

Exclusion criteria included: Karnofsky performance status scale < 70, age greater than 75 years or less than 18 years, complete bowel obstruction or the presence of symptomatic brain metastases, ventricular arrhythmia, congestive heart failure, or documented myocardial infarction, inadequate bone marrow function (WBC count < 3.0 x 109/L or platelet count < 100 x 109/L), inadequate renal function (serum creatinine of no more than 1.25 x upper normal limit or creatinine clearance < 60 mL/min/1.73 m2), and inadequate liver function (serum bilirubin of no more than 1.25 x upper normal limit).

On completion of all of these investigations, the patients of group A were submitted to quadrentectomy & axillary evacuation, while patients of group B were subdivided into 2 groups:

1- FAC group: included 24 patients presented with 25 breast cancers. These patients received neoadjuvant chemotherapy in the form of 3 cycles of FAC regimen (5-flourouracil 500 mg/ m2 I.V. day 1, adriamycin 50 mg/ m2 I.V. day 1, & cyclophosphamide 500mg/ m2 I.V. day 1) with 21 days interval between each two successive cycles.

2- TAC Group: included 8 premenopausal patients with relatively more advanced disease e.g extensive lymph node involvement, with suspicion of presence of micrometastases. They received 3 cycles of TAC regimen (docetaxel 75 mg/ m2 as a one hour I.V. infusion day 1, adriamycin 50 g/m2 I.V. day 1, and cyclophosphamide 500mg / m^2 I.V. day 1) with 21 days interval between each 2 successive cycles. These patients were pre-medicated with 8 mg of oral dexamethasone twice daily for 5 days, starting 1 day prior to docetaxel administration. Growth factor support with granulocyte colony stimulating factor (G-CSF) e.g. fligrastim (Neupogen) was provided to patients who developed neutropenia with or without fever (and was initiated prophylactically to those patients on subsequent cycles).

After each cycle of the neoadjuvant chemotherapy, every patient was examined for the size of the tumour & the regional lymph nodes status. After the third cycle of the neoadjuvant chemotherapy, bilateral mammography & breast ultrasonography were done. The response to the neoadjuvant

chemotherapy was categorized according to

***Dixon et al (1998)* classification:**

-Complete clinical response (CR): complete disappearance of the tumour both clinically & mammographically.

-Partial response (PR): decrease of 50% or more in the total tumour size.

-No response (NR) : decrease of less than 50% or increase of less than 25% in total tumour size.

-Progressive disease (PD): increase of 25% or more in total tumour size.

The patients who responded to induction chemotherapy & downstaged to the extent that make them eligible for breast conservation surgery were submitted to quadrantectomy and axillary evacuation. These patients showed the following tumour characteristics:

1- Complete resolution of skin edema.
2- Residual tumour size of less than 5 cm.
3- No evidence of multicenteric disease.
4- Absence of extensive lymph nodes involvement or extensive microclafication on mammographic examination.

On the other hand, the patients who failed to respond to the neoadjuvant chemotherapy e.g. patients with NR & PD were submitted to modified radical mastectomy (MRM).

Adjuvant treatment:

After removal of the stiches, patients of group A received 6 cycles, while patients of group B received 3 cycles of FAC regimen as an adjuvant therapy.

After completion of chemotherapy, radiotherapy was given using ^{60}Co starting 1 week after the 6th cycle of chemotherapy. Radiotherapy was given to the preserved breast and supraclavicular lymph nodes after conservative breast surgery (CBS) , while it was given to the chest wall and supraclavicular lymph nodes after MRM. The total dose of radiotherapy was 50 Gy for every patient divided into 25 fractions each fraction was 2 Gy given daily for 5 days each week for 5 weeks. After this dose, patients with CBS received a boost dose of 10 Gy delivered to the tumour bed divided into 5 fractions, each fraction was 2 Gy given daily for 5 days.

ER+ve premenopausal patients & all postmenopausal patients (either ER+ve or ER-ve) received tamoxifen in the dose of 20 mg daily for 5 years starting after completion of radiotherapy.

Follow up of the patients:

Every patient was followed up on 3-months basis in the 1st postoperative year & at 6-months intervals later on. On each follow up visit, mammography (bilateral after CBS and on the contralateral breast after MRM), Chest X-ray, pelvi-abdominal ultrasonography were also done. MRI was selectively requested for 1 case to role in or out local recurrence after CBS.

C.A. 15.3 and isotope bone scan was requested for all cases one year after the operation and at one year intervals later on during the follow up period. However, it was requested at shorter intervals if needed.

Results:

The response to the neoadjuvant chemotherapy in group B was assessed both clinically & mammographically. The overall response was 54.5% (18/33). As shown in table 3, the overall response rate in the FAC group was 52% (13/25) while it was 62.5% (5/8) in the TAC group. One patient of those who received the FAC regimen developed complete clinical response. Seventeen patients (12 in the FAC group & 5 in the TAC group) showed partial response; thirteen out of them underwent breast conservation surgery, while the remaining 4 patients (3 in FAC group & 1 in TAC group) showed partial response but not to the degree that make them eligible for breast conservation surgery, so, they underwent modified radical mastectomy. Hence, patients who underwent CBS represented 44% (14/32) of the patients included in group B.

Fifteen patients (12 in FAC group & 3 in TAC group) showed no response (NR). Progressive disease (PD) was not recorded in either regimens.

Table 4 shows that before induction chemotherapy T3 & T4 tumours represented 96% (24/25) in FAC group & 100% (8/8) in

TAC group. This ratio is markedly decreased to 60% (15/25) for the FAC group & 50% (4/8) for the TAC group after induction chemotherapy. This change in the tumour size was found to be statistically significant (P = 0.004 for the FAC regimen & 0.046 for the TAC regimen).

With the use of the neoadjuvant chemotherapy, 10 cases (40%) in FAC group & 4 cases (50%) in TAC group were downstaged to T0, T1,& T2 allowing breast conservation surgery.

Table 5 shows that N2 & N3 tumours represented 24% (6/25) in FAC group & 37.5% (3/8) in TAC group before the neoadjuvant chemotherapy. This ratio was reduced after neoadjuvant chemotherapy to 8% (2/25) in FAC group & 0% (0/8) in TAC group. N0 nodes represented 0% (0/25) before & 16% (4/25) after the neoadjuvant chemotherapy in FAC group, while they represented 0% (0/8) before & 12.5% (1/8) after neoadjuvant chemotherapy in TAC group. N1 nodes represented 76% (19/25) before & the same percentage after neoadjuvant chemotherapy in FAC group, while they represented 62.5% (5/8) before & 87.5% (7/8) after neoadjuvant chemotherapy in TAC group. However, this change in the nodal state was found to be significant only for the FAC regimen (P = 0.007) & not for the TAC regimen (P = 0.059).

Follow up results: With a follow up period ranged from10 to 36 months, treatment failure developed in 1 patient in group A (3.3%) who developed local breast recurrence & submitted to completion mastectomy, while in group B treatment failure occurred in 5 patients (15.5%). Three patients were included in the TAC group: one patient developed lung metastasis, another one developed a new primary cancer in the contralateral breast, & the third one developed chest wall reccurence & opposite breast & bone metastasis. Treatment failure developed in 2 patients of the FAC group: one patient developed chest wall recurrence & the other one developed bone metastasis.

Table 1:Patients Characteristics

Patient characteristic	Group A (No=30)		Group B(No=32 with 33 cancers)	
	No	%	No	%
Age in years				
< 35	5	17	7	22
35- < 45	9	30	9	28
> 45	16	53	16	50
Menstrual status				
Premenopausal	18	60	15	47
Postmenopausal	12	40	17	53
Marital status				
Married	22	73	23	72
single	8	27	9	28
Normal lactation				
+ve	17	57	19	59
-ve	13	43	13	41
Oral contraceptives				
+ ve history	13	43	12	37.5
- ve history	17	57	20	62.5
History of breast lesions				
breast abscess	1	3	0	0
Excision of benign mass	1	3	0	0

Family history + ve - ve	6 24	20 80	5 28	15 85

Table 2:Tumour Characteristics

Tumour characteristic	**Group A (No=30)**		**Group B (No=32 with 33 cancers)**	
	No	%	No	%
Clinical presentation : Breast mass	29	97	32	97
N0	13	43	0	0
N1	17	57	24	73
N 2	0	0	7	21
N3	0	0	2	6
Skin ulceration	0	0	1	3
Skin redness	0	0	2	6
Skin edema	0	0	8	24.5
Laterality: Right breast				
	18	60	23	70
Left breast	12	40	9	27
Bilateral	0	0	1	3
Primary tumour site: Upper outer quadrant				
	19	63	14	42.5
Upper inner quadrant	5	17	8	24.5
Lower outer quadrant	2	7	5	15
Lower inner quadrant	4	13	2	6
Retroareolar region	0	0	2	6
Occupying more than one quadrant	0	0	2	6

Table 3. The clinical response after neoadjuvant chemotherapy in group B

Clinical response	F.A.C. Group	T.A.C. Group	Total
Complete response	1 (4)	0 (0)	**1**
Partial response	12 (48)	5 (62.5)	**17**
No response	12 (48)	3 (37.5)	**15**
Progressive disease	0 (0)	0 (0)	**0**
Total	**25 (100)**	**8 (100)**	**33**

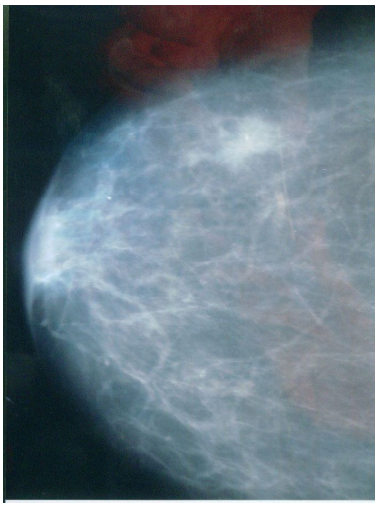
Table 4. Tumour size assessment

Tumour size (T)	**Group A**	**Group B**

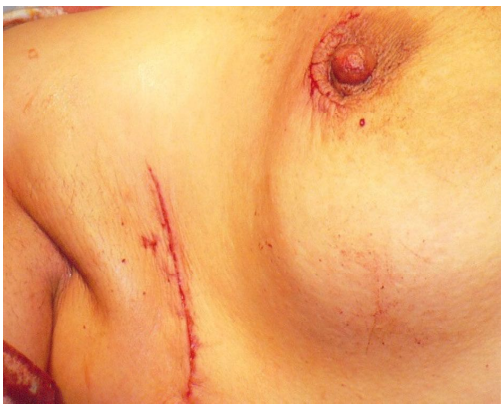
		F.A.C. regimen		T.A.C. regimen	
		Before (%)	**After (%)**	**Before (%)**	**After (%)**
T0	1	1 (4)	2 (8)	0 (0)	0 (0)
T1	14	0 (0)	3 (12)	0 (0)	3 (37.5)
T2	15	0 (0)	5 (20)	0 (0)	1 (12.5)
T3	0	16 (64)	10 (40)	5 (62.5)	2 (25)
T4	0	8 (32)	5 (20)	3 (37.5)	2 (25)
Total	**30(100)**	**25 (100)**	**25 (100)**	**8 (100)**	**8 (100)**

Table 5. Nodal state assessment

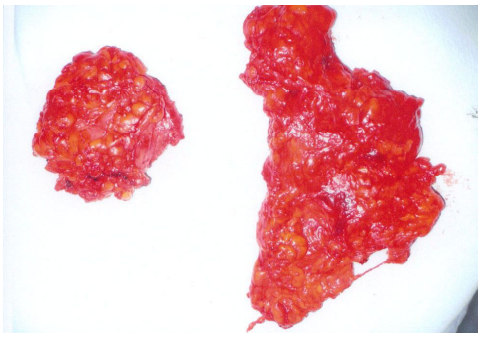
Nodal State (N)	**Group A(%)**	**Group B**			
		F.A.C. regimen		**T.A.C. regimen**	
		Before (%)	**After (%)**	**Before (%)**	**After (%)**
N0	13(43)	0 (0)	4 (16)	0 (0)	1 (12.5)
N1	17(57)	19 (76)	19 (76)	5 (62.5)	7 (87.5)
N2	0	5 (20)	2 (8)	2 (25)	0 (0)
N3	0	1 (4)	0 (0)	1 (12.5)	0 (0)
Total	**30(100)**	**25 (100)**	**25 (100)**	**8 (100)**	**8 (100)**



- Figure 1: A mammogram shows T1 tumour.



- Figure 2: Incisions for conservative breast surgery.



- Figure 3: The removed specimen from the breast & the axilla.

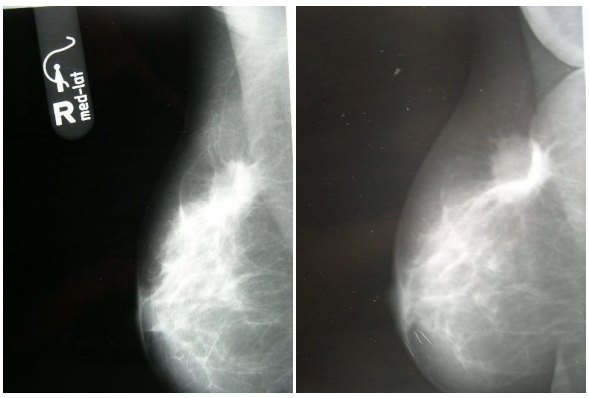


Figure 4a Figure 4b

- Figure 4: a- A mammogram of breast mass before neoadjuvant treatment
b- A mammogram of the same mass after neoadjuvant treatment with partial response

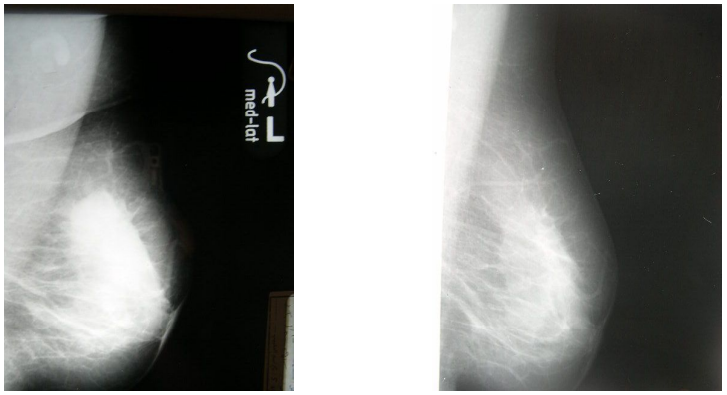


Figure 4a Figure 4b

- Figure 5: a- A mammogram of breast mass before neoadjuvant treatment
b- A mammogram of the same mass after neoadjuvant treatment with complete response

Discussion:

Breast conserving therapy (BCT) including postoperative irradiation of the remaining breast tissue is generally accepted as the best treatment for the majority of patients with early-stage breast cancer *(Tinterri et al, 2009).* In recent years, an increasing number of patients with locally advanced breast cancer (LABC) are being treated with neoadjuvant chemotherapy, followed by breast conservation surgery with axillary dissection and radiation as a part of the multimodality management *(Tewari et al, 2009).*

Neoadjuvant chemotherapy is now accepted to be a standard milestone in the treatment of locally advanced breast cancer as it induces down staging in a significant proportion of cases and renders inoperable cases amenable for curative resection (*El-Didi et al., 2000*).

In the present study, patients age ranged between 27 & 69 years with a mean age of 47.2±10.44 years. The premenopausal patients represented 53.2% of all the patients. Pierga et al (2000) reported a mean age of 47 years & 75% of their patients were premenopausal, while Yoshimoto et al (2004) reported a mean age of 53 years & only 44.6% of the patients were premenopausal. Neoadjuvant chemotherapy was given to 32 patients in the present study. The overall response rate to neoadjuvant chemotherapy was 54.5%; one patient with CR (3%) & 17 patients with PR (51.5%). These results are consistent with *Kim et al (2004)* who reported that the overall response rate to neoadjuvant chemotherapy is 60% (4% CR & 56% PR). However, extreme reports came from *Ciarmiello et al (1998)* who reported a low overall response rate of only 38.5%, & *Abraham et al (1996)* Who reported a high overall response rate of 83% (28% CR & 55% PR). The tumour shrinkage after neoadjuvant chemotherapy was statistically significant for both FAC (P = 0.004) & TAC (P = 0.046) regimens. The change in the nodal state was statistically significant only in the FAC regimen (P = 0.007).

After neoadjuvant chemotherapy, 14 patients became suitable candidates for breast conservation surgery. These patients represent 44% of the patients who received neoadjuvant chemotherapy. These results are supported by *Rouzier et al (2004)* who used anthracycline-based neoadjuvant chemotherapy for 594 patients with invasive breast cancer who were ineligible for breast conservation surgery & they found that 287 (48%) of them became eligible for breast preservation. However, lower figures were reported by other authors. *Danforth et al (1998)* conducted their study on 126 patients with locally advanced breast cancer who received neoadjuvant chemotherapy. They found that 42 (33%) of them were downstaged to the extent that breast conservation surgery became a feasible technique for them. In another study, *Hortobagyi et al (2000)* reported that only 23% of patients with locally advanced breast cancer are good candidates for breast conservation surgery after neoadjuvant chemotherapy provided that they are carefully selected.

Liu et al (2009) reported 5-year local relapse-free rate of 98.3% for patients presented with early breast cancer. This is compatible with the results of our study that showed a local treatment failure rate of 3.3% for group A patients. However, some other studies showed a higher local relapse rates like Nasr et al (2009) who reported local failure rate of 14.3%.

In the present study, 5 patients (15.5%) of group B developed treatment failure during the follow up period. These results are comparable to those of Shen et al (2004) who conducted a study on 33 patients with stage IIIB & IIIC breast cancer treated with neoadjuvant chemotherapy followed by lumpectomy & they found that the 5-year disease-free survival was 70%. Also, Inaji et al (2002) found local recurrence rate of 4.7% after conservative breast surgery in patients with locally advanced breast cancer.

The results of this prospective study demonstrated that conservative breast surgery could be performed safely for patients presented with early breast cancer as well as those presented with locally advanced breast cancer after down staging with neoadjuvant chemotherapy. Neoadjuvant chemotherapy

has significant anti-tumour activity, and it increases the ability to perform breast conservation surgery with the same overall & disease free survival rates as the adjuvant chemotherapy.

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Immunohistochemical Study Of Protein P53 In Egyptian Psoriasis

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Abstract: Background: The histopathologic changes characteristic of psoriasis might be related to an abnormality in the apoptotic pathway.

Aim of the work: The aim of this study is to evaluate the possible role of protein P_{53} in the pathogenesis of psoriasis through a case control study as it could be one of the targets of psoriasis therapy.

Patients and Methods: This study included; 30 patients of different clinical variants of psoriasis and 25 controls normal skin biopsies. All patients were subjected to complete history taking, clinical examination including psoriasis area and severity index (PASI) score and skin biopsies, all patients stopped topical or systemic medication 4 weeks prior to biopsies.Five mm incisional biopsy specimens were taken from the 30 patients and from each biopsy one stained with hematoxylin and eosin to confirm the diagnosis ,the other to be prepared for immunohistochemical detection using mouse monoclonal antibody (Do7) against P53 protein , results were compared with 25 control ..

Results: Psoriatic plaques revealed P53 nuclear staining detected in 13 out of the 30 patients (43.3%), and 17 (56.7%) showed negative immunoreactivity in keratinocytes.

Conclusion: From these results it can be concluded that apoptosis plays a role in the pathogenesis of psoriasis and this may be mediated through abnornal expression of apoptosis regulating proteins P53.

Key words: Apoptosis , immunohistochemistry, P53, Psoriasis.

Introduction

P 53 Gene

P53 is a tumor suppressor gene located on short arm of chromosome (17 p13.1) (Stoll et al., 1998). The gene product is a phosphonucleoprotein composed of 393 amino acids (Shimizu et al., 1997). The wild type P53 seems to negatively regulate cellular proliferation by controlling the cell cycle. Following DNA damage P53 arrest the cell cycle at G1 phase to allow time for DNA repair before entering to S phase. If the damage is extensive enough, P53 initiates programmed cell death or apoptosis. Loss of these various molecular check points has been found to under lie the development of many tumors because cell cycle progression

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becomes unchecked and tumorigenesis results (Meng and El Deiry, 1998). Mutations in P53 gene are the most common genetic alterations in tumors being mutated or deleted in over 50% of all human cancers (Greenblatt et al., 1994).

Since the normal wild type P53 protein has a very short half-life, its concentration is generally below immunohistochemical detection level. Conversely mutant P53 protein has longer half life which makes it detectable (Shimizu et al., 1997), thus it was hypothesized that detectable levels of P53 in tissue implied that P53 was mutant type (Levine et al., 1992). Hall et al., (1993) demonstrated that wild type P53 was hardly detectable in normal epidermis, also Soini et al., (1994) did not find any P53 positive keratinocyte in normal epidermis.

At the molecular level over expression of growth factors and proinflammatory cytokines have been described in psoriatic plaques. On the other hand, the loss of inhibitory control mechanisms are involved in the pathogenesis of the disease as exemplified by the reduced mRNA levels for the cell cycle inhibitor, P53 was found in lesional skin (Michel et al., 1997).

Tumor suppressor genes are frequently altered by mechanisms such as point mutations, deletions of coding or regulatory gene sequences, Transcriptional silencing of genes by hypermethylation, altered protein expression or expression of inhibitory proteins Many of the multiple functions of P_{53} including the primary role of P_{53} tumor suppression, can be attributed to its ability to act as a sequence – specific transcription factor which regulates expression of different cellular genes to modulate various cellular processes. (Francis et al., 2007).

As a cellular gate keeper, one of roles of p53 is to monitor cellular stress and induce apoptosis as necessary (Hofseth et al., 2004). In tissues where stressors generate severe and irrevocable damage, p53 can initiate apoptosis, Thereby eliminating damaged cells. (Sogame et al., 2003). The products of these genes may induce apoptosis through either an intrinsic pathway (The mitochondrial pathway) or an extrinsic pathway (The death receptor pathway). (Cory et al., 2002) or through transcription independent mechanics (Haupt et al., 2003). The p53 tumor suppressor protein , integrates diverse physiological signals in mammalian cells.

In response to stress signals, perhaps the best studied of which is the response to DNA damage, p53 becomes functionally active and triggers either a transient cell cycle arrest, cell death (apoptosis) or permanent cell cycle arrest (cellular senescence) .Both apoptosis and cellular senescence are potent tumor suppressor mechanisms that irreversibly prevent damaged cells from undergoing neoplastic transformation (Al Rashid et al., 2005).

In most cell types, activation of p53 is crucial for initiating the senescence response following DNA damage. In some cells, p53 is also important for maintaining the senescence growth arrest. In others, p53 is required only to establish the senescence growth arrest, which subsequently becomes irreversible and p53-Independent (Beausejour et al., 2003).

Psoriasis

The knowledge gained over the past 3 decades enables researches to postulate that psoriasis is a T-cell immune – mediated skin disease in which various cells play a dominant role at different stages (Sabat et al., 2007). In most reviews, the prevalence of psoriasis is said to be 2% of the world's population. Psoriasis can first appear at any age. Two peaks in age of onset have been reported: one at 20 – 30 years of age and a second peak at 50 – 60 years. In approximately 75% of patients, the onset is before the age of 40 years (Griffiths et al., 2004).

The clinical spectrum of psoriasis is a chronic recurring skin condition which varies in severity from minor localized plaques to complete body coverage. Fingernails and toenails are frequently affected (psoriatic nail dystrophy) and can be seen as an isolated finding. Psoriasis can also cause inflammation of the joints known as psoriatic arthritis (Helliwell and Taylor, 2005).

Different clinical types of psoriasis exist. These include plaque type psoriasis, guttate psoriasis, psoriasis of the palms and soles, facial psoriasis, flexural psoriasis, pustular psoriasis, erythrodermic psoriasis,

nail psoriasis, genital and oral psoriasis.

Psoriasis represents a T-cell-mediated inflammatory skin disease, although the primary pathogenic mechanism is still unknown (Ghoreschi et al., 2007). Specific T-cell populations are stimulated by putative antigen presented by antigen presenting cell (APC) from the skin. This T-cell /APC ("T-AP") interaction is much like a tap-dance where specific steps must occur in sequence to result in T-cell activation; otherwise T-cell anergy would occur (Fearon and Veale, 2001).The knowledge gained over the past 3 decades enables researches to postulate that psoriasis is a T-cell immune – mediated skin disease in which various cells play a dominant role at different stages (Sabat et al., 2007)

It has been recognized that an association between human immunodeficiency virus (HIV) infection and psoriasis exists in the form of induction or exacerbation of psoriasis (Maurer, 2006). There are many drugs reported to be responsible for the onset or exacerbation of psoriasis (Abel et al., 1986). Such as lithium salts, antimalarials, β- blocking agents, non-steroidal anti-inflammatory drugs (NSAIDS), angiotensin-converting enzyme (ACE) inhibitors and the withdrawal of corticosteroids.Although sunlight is generally beneficial, in a small minority of patients, psoriasis may be provoked by strong sunlight and cause summer exacerbation in exposed skin (Griffiths et al., 2004).

The early onset of psoriasis in women, with a peak around puberty, changes during pregnancy and provocation of psoriasis by high- dose estrogen therapy potentially indicates a role for hormonal factors in the disease (Griffiths et al., 2004).Psoriasis has a major effect on the psychological quality of life patients and the stress management programmes significantly shorten the time to clearance with standard therapies (Fortune et al., 2003). Studies suggest that alcohol may exacerbate pre existing disease but doesn't appear to induce psoriasis (Rosset and Oki, 1971). Alcohol may also reduce the therapeutic compliance or may reflect a symptom of stress caused by severe psoriasis (Behnam et al., 2005).

Aim of the work

The aim of this study is to evaluate the possible role of protein P_{53} in the pathogenesis of psoriasis through a case control study as it could be one of the targets of psoriasis therapy.

Patients and Methods

This study included 30 patients of different clinical variants of psoriasis and 25 controls. The patients group consisted of 16 male and 12 females with their age ranging between 18 to 60 years. They were selected from dermatology outpatient clinic of Ain – Shams University Hospital and National research center during the period between August 2008 to August 2009.

Ethical considerations:

For confidentiality, the names were excluded and replaced with a numerical code.

A) Full history taking:

1. Personal history:

Name, age, sex, occupation and residence.

2. History of the disease:

• Age of onset, course and duration of the disease.
• Exacerbating and relieving factors.
• History of previous treatment.
• Family history of similar condition.
• Past history of other systemic affection

B) Clinical examination:

Site and severity of skin involvement.

Distribution of the lesion, generalized or localized.

Nail, scalp and joint affection.

Extent of skin involvement.

C) Clinical assessment:

Clinical assessment of psoriatic patients was performed through calculation of psoriatic area and severity index (PASI score) **(Fredriksson and Pettersson., 1978).**

PASI score:

For calculation of this index: the four main body areas were assessed: the head (h), the trunk (t), the upper extremities (u), and the lower extremities (I) corresponding to 10, 20,

30 and 40% of the total body area, respectively.

Three target symptoms namely erythema (E), infiltration (I) and desquamation (D) were assessed according to scale 0-4 where means complete lake of cutaneous involvement and 4 represents the severest possible involvement.

Investigation

5 mm incisional biopsies' were taken from sun unexposed lesions of psoriatic patients as well as from normal control. the biopsies were fixed in 10% neutral buffered formalin and paraffin embedded blocks were prepared

Two slides were made from each biopsy:

One slide was stained by hematoxylin and eosin stain and was examined by light microscopy to confirm the diagnosis.

The other slide was cut over positive charged coated slides for immunohistochemical staining using monoclonal antibody against p53 protein by immunoperoxidase technique

Immunohistochemical study:

The kit used for immunohistochemical staining was “supersensitive ready to use immunodetection system" presented by clini lab. The kit contained P53 monoclonal antibody clone D07 which detects nuclear localization of P53 protein. This antibody reacts with both wild type and mutant type of P53 protein.

The staining procedure:

• The paraffin embedded sections were cut over positive charged coated slides produced by clini lab.

• The slides were then immersed in xylol over night.

•The slides were rehydrated by immersion in alcohol with descending concentrations (5 minutes in two changes of 100% ethanol, 5 minutes in two changes of 95% ethanol) to wash the xylol and remove any impurities, then they were washed with water

•They were immersed in buffer solution pH 7.4 for 5 minutes. The buffer was formed of sodium chloride + Potassium phosphate monobasic + potassium dibasic.

•Endogenous peroxidases were inhibited by treating the slides with 3% hydrogen peroxide in distilled water for 5 minutes.

• The slides were washed in buffer and were-wiped around the section.

•Antigen retrieval citra 10% (also produced by clini Lab) was then added to the sections to unmask hidden or weak antigens in tissues.

•The slides were put in a bath of water and microwaved for 5 minutes. They were removed from the microwave and inspected to see if the level of water around the slides has decreased. Water was then added to cover the slides if it was not enough and antigen retrieval was added to the sections once more, then they were remicrowaved for another 5 minutes.

• The slides were left to cool for 15 minutes.

• The slides were washed with water then with buffer.

•The primary antibody was then added to the slides which were monoclonal antibody clone D07 which reacts with both wild type and mutant form of P53 protein.

• The slides were incubated for one hour in a humid chamber.

• The slides were washed in buffer.

•The link pre-diluted biotyonylated anti-immunoglobulin was added for 20 minutes.

•Then the slides were washed in buffer and were wiped around the sections.

•Slides were then labeled by peroxidase conjugated streptavidin for 20 minutes.

• Slides were washed again in buffer and wiped around the sections

•Liquid DAB (3,3 diaminobenzidine) chromagen was then added with H_2O_2 (as substrate) for 20 minutes to visualize immunoreactivity sites.

•The slides were washed with distilled water and wiped around the sections.

•Counter stain was added (Mayer's hematoxylin) for 1 to 2 minutes.

•Slides were then washed in water and passed through rising concentrations of alcohol.

• They were then put in xylen for 5 minutes.

• Finally Canada balsm was added then the slides were cover.

• All through the procedure slides were not allowed to dry.

• The slides were then read under the light microscope.

Interpretation of the results of immunostaining:

Positive staining appeared as brown coloration of the nuclei of variable intensities (mild, moderate and strong).

Criteria of staining:

A semi quantitative score was applied to the sections as follows:

• Negative (-): if no cells were stained.

• Mild expression (+): if less than 10% of the cells were stained.

•Moderate expression (++): if 10% to 50% of the cells were stained.

•Strong expression (+++): if more than 50% of the cells were stained.

Results

This study included 30 patients with different clinical variants of psoriasis. They were 16 males (53.3%) and 14 females (46.7%) and their age ranged between 18 to 60 years with mean ± SD (36.3 ± 9.7). The duration of psoriasis ranged between 1 to 16 years with mean ± S.D (6.8 ± 4.2). The psoriatic area and severity index (PASI) ranged between 3.3 to 67.6 with mean ± S.D (22.6 ± 22.2).Three of the 30 patients (10%) had positive family history of psoriasis . Thirteen psoriatic patients showed +ve P53 expression (Table 1). Skin biopsies from 25 apparently healthy individuals were taken and served as control after their consent. They were 15 males (60%) and 10 females (40%). Their age ranged between 16 to 64 years with mean ±S.D (35.5 ± 13.7), all were negative for P53 expression (Table 2).

Table (3) & figure (1) show no statistically significant difference between psoriatic group and control group as regards sex, as P > o.o5.Table (4) & figure (2) show no statistically significant difference between psoriatic group and control group as regards age , as P > o.o5

Table (5) shows a comparison between different clinical types of psoriasis and intensity of staining of P53. Five patients (16.6%) with psoriasis vulgaris, 2 of which (40%) show negative intensity of staining of P53 and 3 of which (60%) show mild P53 expression. Five patients (16.6%) with guttate type psoriasis all of them (100%) show negative P53 expression. Five patients (16.6%) with psoriasis of palm and sole, 1 of which (20%) show negative P53 expression and 4 of which (80%) show mild P53 expression. Five patients (16.6%) with nail psoriasis, 4 of which (80%) show negative P53 expression and 1 of which (20%) show mild P53 expression. Five patients (16.6%) with flexural psoriasis all of them (100%) show negative P53 expression. Five patients (16.6%) with erythrodermic psoriasis, 2 of which (40%) show mild P53 expression and 1 patient (20%) shows moderate P53 expression and 2 patients (40%) show strong P53 expression. These findings mean that there is strong correlation between the severity of psoriasis and the intensity of staining of P53.

Table (6) & figure (3) showed that 6 (46.2%) psoriatic patients out of 13 (100%) psoriatic patients who showed +ve P53 expression were males while 7 (53.8%) psoriatic patients out of 13 (100%) showed + ve P53 expression were females. In figure (4), only 2 psoriatic patients out of 13 who were P53 +ve have +ve family history while 11(84.6%) psoriatic patients out of 13 who were P53 +ve have –ve family history.

Table (8) & figure (5) show positive correlation between PASI score and the duration of psoriasis. Table (9) & figure (6) show a correlation between PASI score and P53 expression which was significant.

In table (10) & figure (7), the comparison between psoriatic group and control group as regards to P53 expression showed that all the 25 control healthy individuals were P53-ve, while among the psoriatic group, 17 (56.7%) showed negative P53 expression, while 10(33.3%) showed mild P53 expression, 1 (3.3%) showed moderate P53 expression, 2 (6.7%) showed strong P53 expression.

Table (11)& figure (8) Showed that there is 13 psoriatic patients had +ve intensity of P53 staining, 10 (76.9%) of which showed mild intensity of staining, 1 (7.7%) of which showed moderate intensity of staining, 2

(15.4%) of which showed strong intensity of staining.

Table (12) show that the age of those patients with + ve P53 expression was higher compared to those with – ve P53 expression and this difference was statistically significant (P < o.o5). Table (13) & figure (9) showed that the duration of the disease was much higher among patients with +ve P53 expression compared to that with –ve P53 expression and the difference was statistically significant (P < o.o5).

Table (1) CLINICAL AND DEMOGRAPHIC DATA FOR PSORIATIC PATIENTS AND P53 EXPRESSION.(n = 30)

No	Age/year	sex	Duration/ year	FH	Types of psoriasis	PASI	P53 expression
1	24	F	3	Negative	V	10.9	**-ve**
2	31	F	8	Negative	V	13.7	**-ve**
3	38	M	10	Negative	V	31.3	**+1**
4	42	F	10	Negative	V	25.9	**+1**
5	60	M	6	Negative	V	15.3	**+1**
6	38	M	4	Negative	G	27.4	**-ve**
7	44	M	8	Negative	G	35.2	**-ve**
8	39	M	12	Negative	G	29.12	**-ve**
9	40	M	4	Negative	G	41.4	**-ve**
10	31	F	11	positive	G	39.5	**-ve**
11	41	M	2	Negative	P	4.5	**-ve**
12	33	M	3	Negative	P	8.8	**+1**
13	37	F	6	Negative	P	10.9	**+1**
14	29	F	3	Negative	P	9.7	**+1**
15	34	F	5	Negative	P	6.9	**+1**
16	22	M	5	Negative	N	3.6	**-ve**
17	31	F	9	Negative	N	4.5	**+1**
18	34	M	10	Negative	N	3.7	**-ve**
19	29	F	3	Negative	N	4.2	**-ve**
20	40	F	1	Negative	N	3.3	**-ve**
21	22	M	4	Negative	F	4.8	**-ve**
22	25	M	2	Negative	F	3.3	**-ve**
23	33	M	5	Negative	F	5.7	**-ve**
24	37	F	1	Negative	F	9.4	**-ve**
25	18	F	6	Negative	F	6.9	**-ve**
26	44	F	10	positive	E	62.3	**+1**
27	52	M	16	Negative	E	67.6	**+3**
28	39	F	14	Negative	E	60.1	**+1**
29	50	M	9	Negative	E	64.4	**+3**
30	52	M	15	Positive	E	66.8	**+2**

M = Male , **F** = Female , **FH** = Family History ,**PASI** = Psoriatic Area and Severity Index.

Intensity of staining of P53: (P53 expression):

Negative (-) ;Mild Expression (+ 1);Moderate Expression (+ 2);Strong Expression (+ 3)

Types of psoriasis:

V = Psoriasis Vulgaris; **G** = Guttate Psoriasis **;P** = Psoriasis of Palms and Soles; **N** = Nail

Psoriasis
E= Erythrodermic Psoriasis

Table (2) = CONTROL GROUP AND P53 EXPRESSION(n = 25)

No	age	sex	P53 expression
1	22	M	Negative
2	33	F	Negative
3	16	M	Negative
4	21	M	Negative
5	24	M	Negative
6	33	M	Negative
7	32	M	Negative
8	40	F	Negative
9	52	F	Negative
10	19	F	Negative
11	33	M	Negative
12	44	M	Negative
13	22	F	Negative
14	39	M	Negative
15	21	M	Negative
16	30	M	Negative
17	23	M	Negative
18	27	M	Negative
19	31	M	Negative
20	55	F	Negative
21	57	F	Negative
22	53	F	Negative
23	47	F	Negative
24	64	M	Negative
25	49	F	Negative

M= Male F=female

Table (3): Comparison between psoriatic group and control group as regards sex:

Sex / Group	Male		Female		Total	X^2	P
	No.	%	No.	%			
psoriatic group	16	53.3%	14	46.7%	30	0.3	>0.05
Control group	15	60%	10	40%	25		
Total	31	56.4%	24	43.6%	55		

P > 0.05 = non significant .P < 0.05 = significant

%

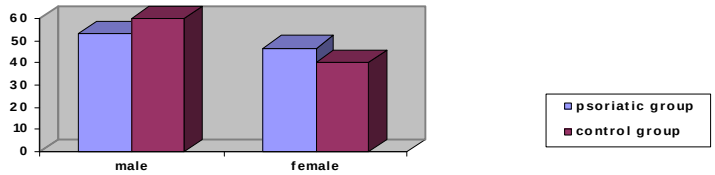


Figure (1) : Comparison between psoriatic group and control group as regards sex

Table (4): Comparison between psoriatic group and control group as regards age:

Group / Age	psoriatic group	control group	t	P
_Age (X±SD)	36.3±9.7	35.4±13.7	0.3	>0.05

P > 0.05 = non significant .P < 0.05 = significant

Years

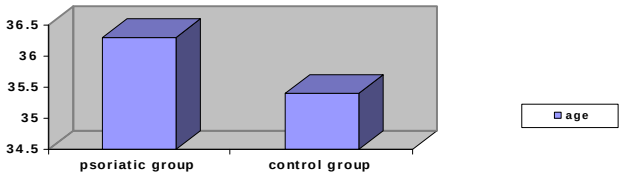


Figure (2): Comparison between psoriatic group and control group as regards age:

Table (5): Comparison between different types of psoriasis and intensity of staining of p53:

Stain intensity / Type	Negative		Mild		Moderate		Strong		Total
	No.	%	No.	%	No.	%	No.	%	
Psoriasis vulgaris	2	40	3	60	0	0	0	0	5
Guttate psoriasis	5	100	0	0	0	0	0	0	5
Psoriasis of palm and sole	1	20	4	80	0	0	0	0	5
Nail psoriasis	4	80	1	20	0	0	0	0	5
Flexural psoriasis	5	100	0	0	0	0	0	0	5
Erythrodermic psoriasis	0	0	2	40	1	20	2	40	5
Total	17	56.7	10	33.3	1	3.3	2	6.7	30

Table (6): Comparison between male and female cases as regards to p53 expression:

	Number	%
Male	6	46.2%
Female	7	53.8%
Total	13	100%

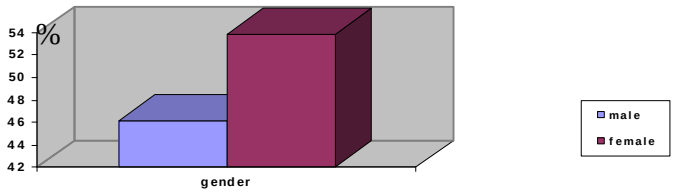


Figure (3): Comparison between male and female cases as regards to p53 expression:

Table (7): Comparison between family history as regards to p53 expression:

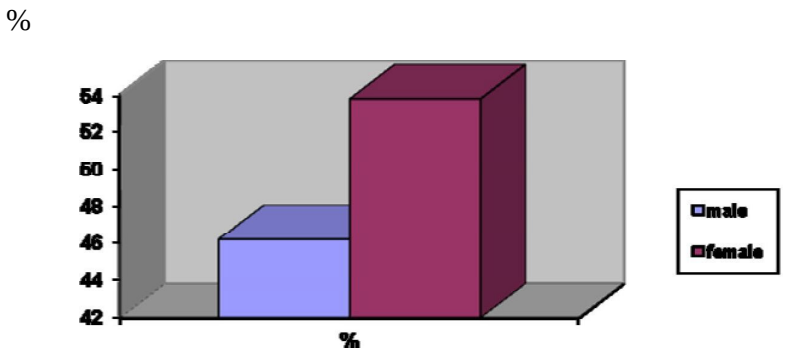
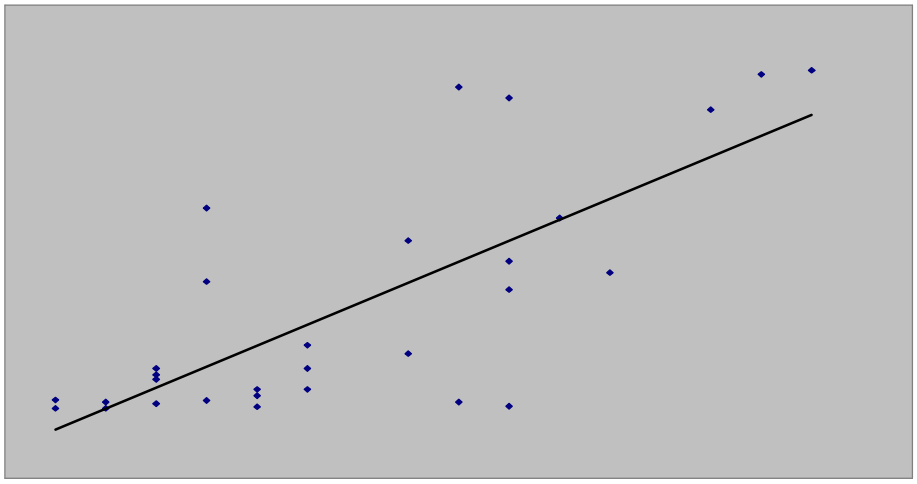


Figure (4) : Comparison between family history as regards to p53 expression

Table (8): Correlation between PASI score and duration of psoriasis

	R	P
Duration of psoriasis	0.75	<0.05

P > 0.05 = non significant .P < 0.05 = significant



PASI score

Duration in years

Figure (5): Correlation between PASI score and duration of psoriasis

Table (9): Correlation between PASI score and p53 expression:

	R	P
P53 expression	0.65	<0.05

P > 0.05 = non significant .P < 0.05 = significant

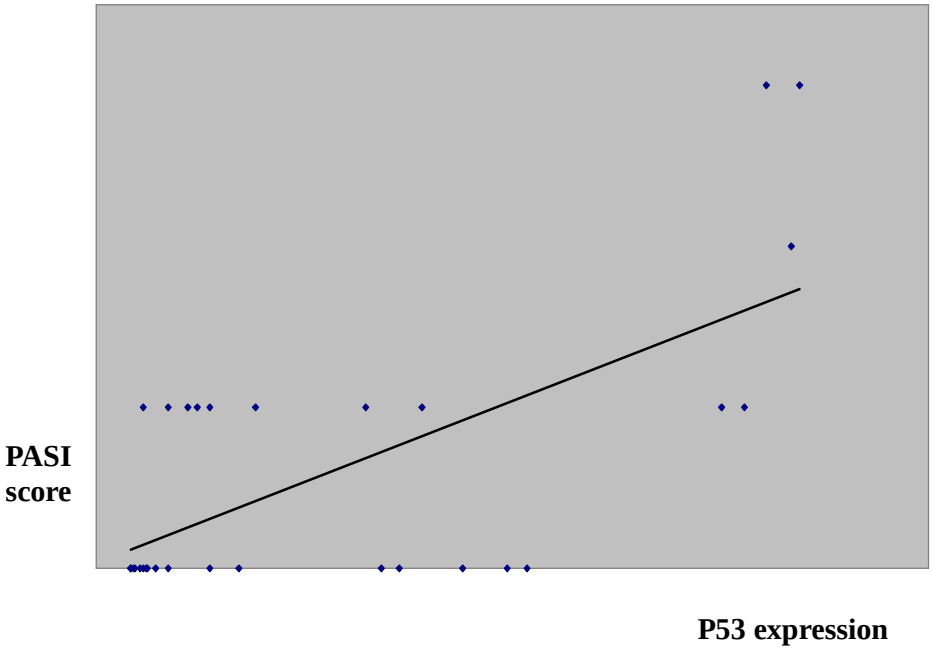


Figure (6) : Correlation between PASI score and p53 expression

Table (10): Comparison between psoriatic group and control group as regards to p53 expression:

Group / Expression	Psoriatic group		Control group	
	No.	**%**	**No.**	**%**
Negative	17	56.7%	25	100%
Mild	10	33.3%	0	0%
Moderate	1	3.3%	0	0%
Strong	2	6.7%	0	0%
Total	30	100%	25	100%

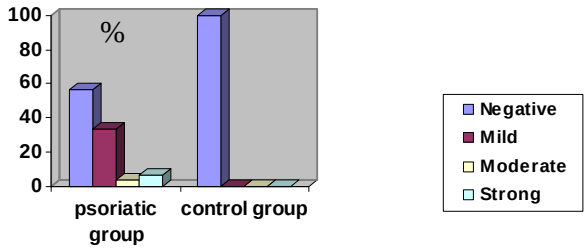


Figure (7) : comparison between psoriatic group and control group as regards to p53 expression:

Table (11): the intensity of staining of p53 among positive group of psoriatic patients:

	Psoriatic group	
	No.	**%**
Mild	10	76.9%
Moderate	1	7.7%
Strong	2	15.4%
Total	13	100%

:

%

Figure (8) : the intensity of staining of p53 among positive group of psoriatic patients

Table (12): comparison between positive and negative P53 as regards to age:

	Positive P53	**negative P53**	**T**	**P**
Age (X±SD)	41.6±9.5	32.2±7.9	2.9	<0.05

P > 0.05 = non significant .P < 0.05 = significant

Table (13): comparison between positive and negative P53 as regards to duration:

P53 positivity / **Duration**	**Positive P53**	**negative P53**	**T**	**P**
($\overline{X}$±SD)	8.9±4.2	5.3±3.4	2.6	<0.05

P > 0.05 = non significant .P < 0.05 = significant

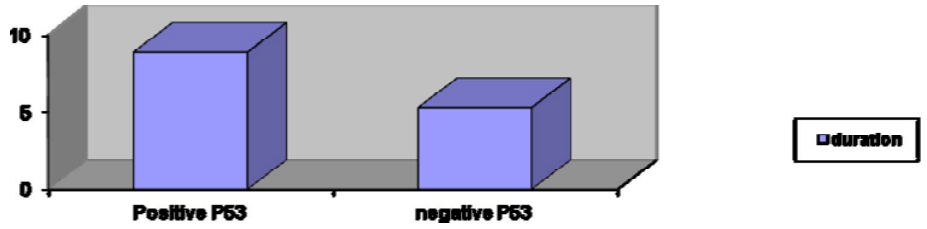


Figure (9) : comparison between positive and negative P53 as regards to duration: Light microscopy of psoriasis

The fully developed lesions showed :

Regular elongation of the rete ridges with thickening in their lower portion (clubbing).
Elongation and edema of the papillae .
Thinning of the supra papillary portions of the stratummal-pighii, with occasional presence of a very small spongiform pustule of kogoj.
Absence of granular cells.
Parakeratosis .
The presence of Munro micro absences in the parakeratotic horny layer

Immunohistochemical results of P53 expression:

Psoriatic group:

P53 nuclear staining was detected in 13 out of the 30 patients (43.3%) ; while the other 17 (56.7%) showed negative immunoreactivity (Table 5). The expression ranged from mild (10 cases, 76.9%) to moderate (1 case, 7.8%), to strong expression (2 cases, 15.4%). (Table 5)& (figure 12 to 16).

Control group (figure 10): shows normal skin, all were negative for P53 expression.

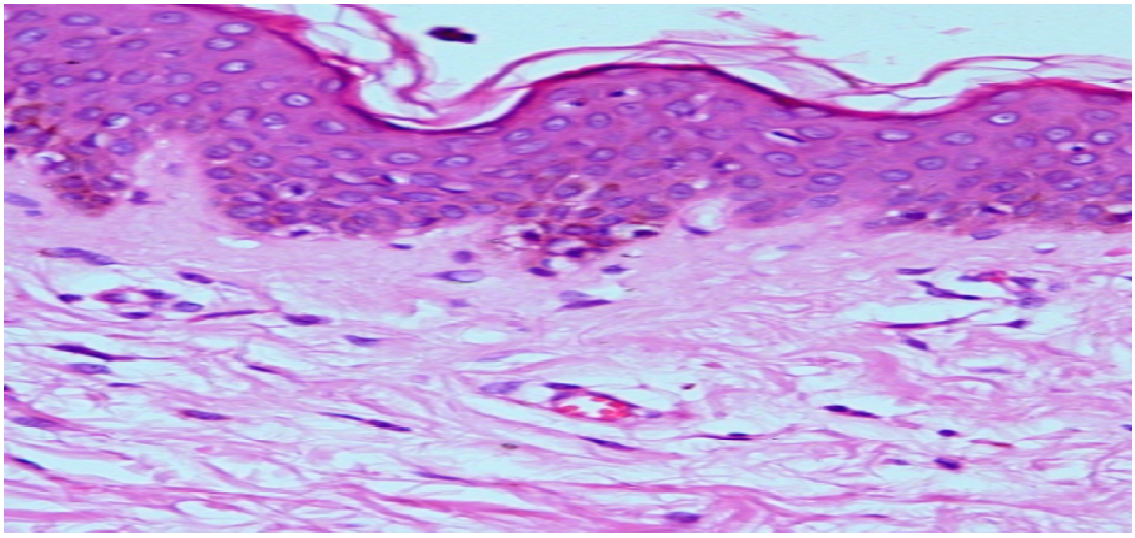


Figure (10) : Healthy skin control (x200)

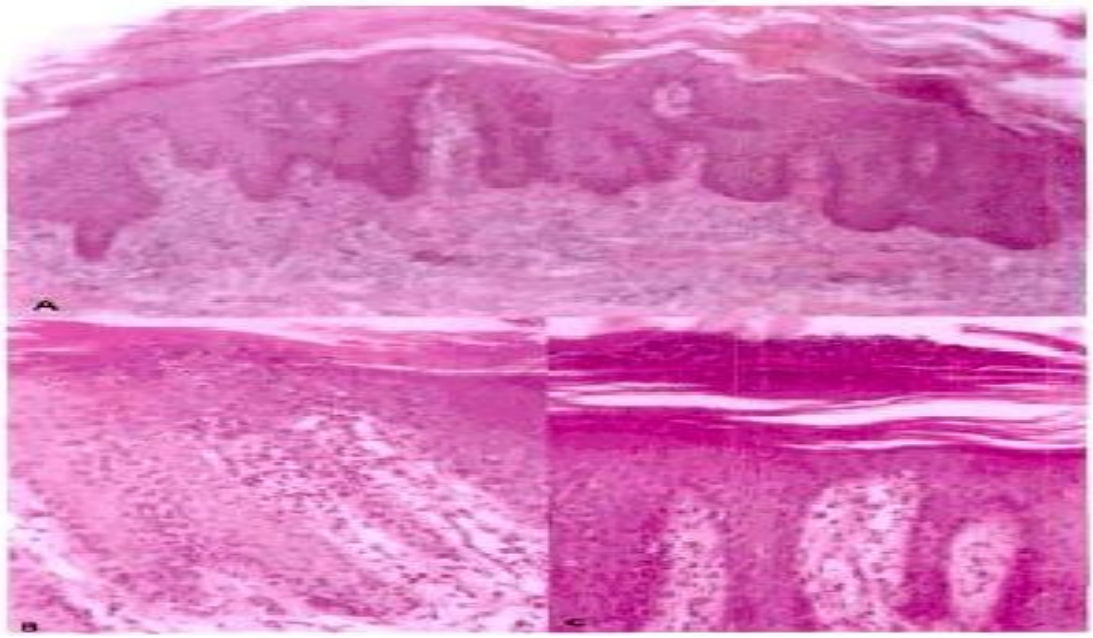


Fig. (11). Psoriatic plaque showing parakeratosis,epidermal hyperplasia, clubbed Rete ridges and lymphocytic infiltrate(A). Upper epidermis shows absent granular layer and

spongiform pustule of Kogoj.Papillary dermis shows dilated blood vessels and lymphocytic infiltrate(B). Munro microabcesses within the stratum corneum(C) (H&E, XI00 in A; X200 in B and C)

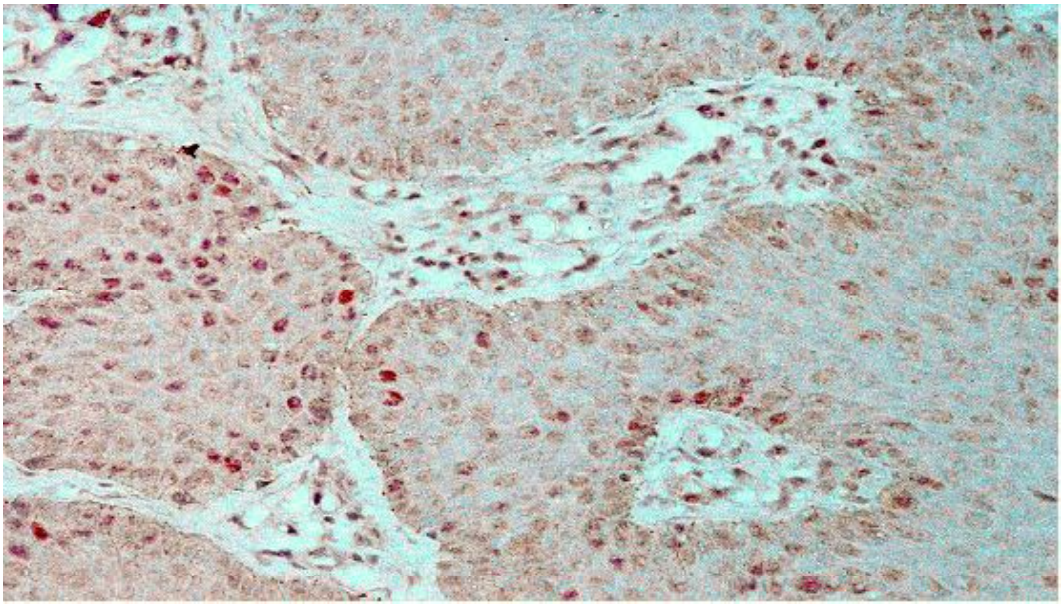


Figure (12) :Lesional psoriatic skin with Strong nuclear +3 immunoreactivity P53 expression in suprabasal keratinocytes ((x200)

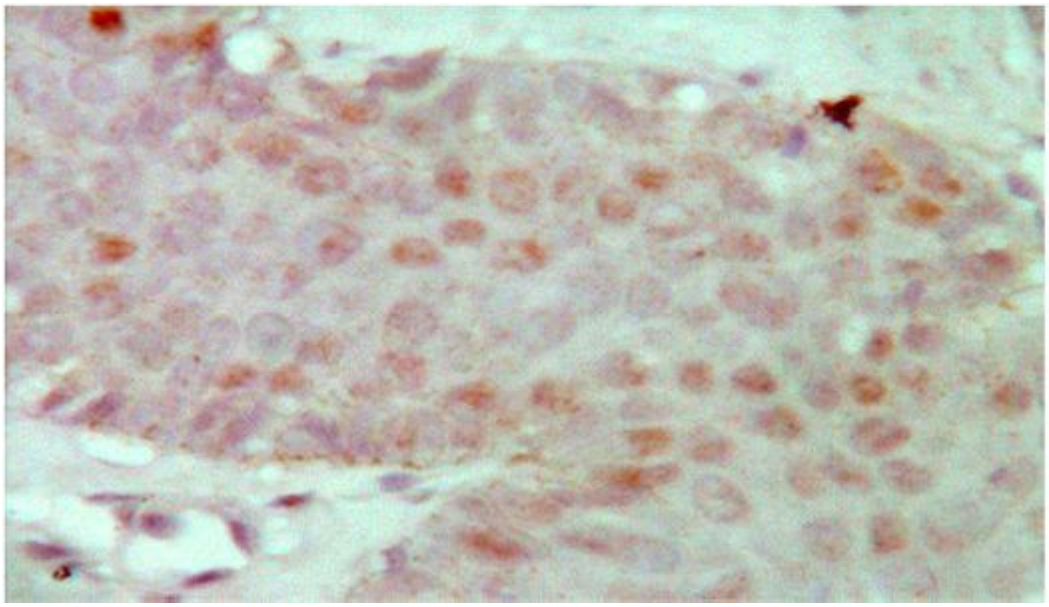


Figure (13):Lesional psoriatic skin showing epidermal hyperplasia with Strong nuclear +3 immunoreactivity P53 expression in suprabasal keratinocytes (x400)

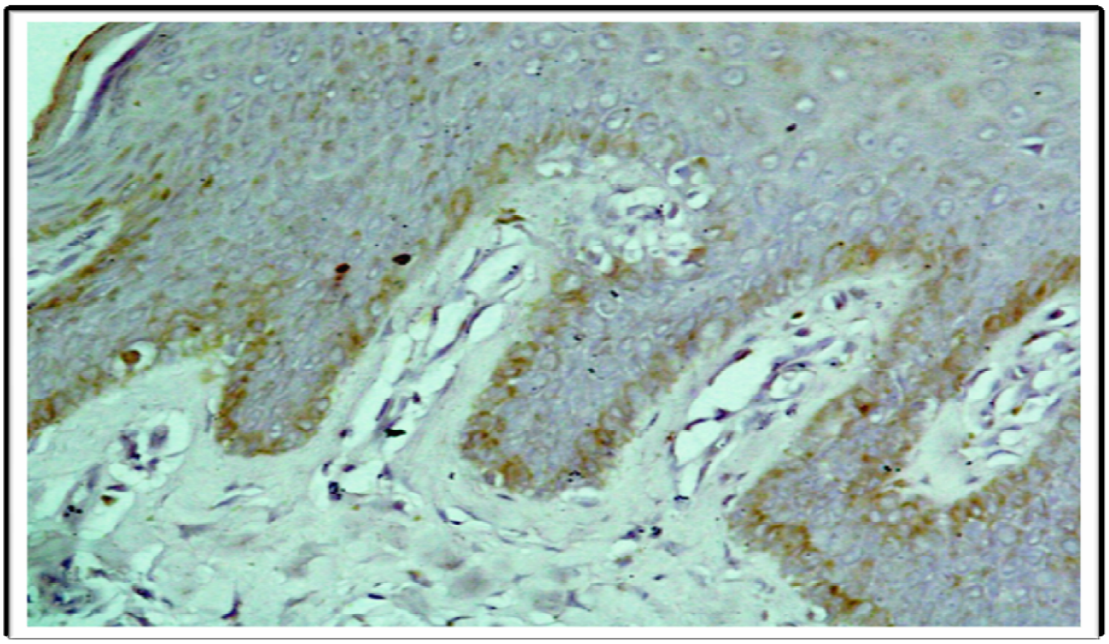


Figure (14):Lesional psoriatic skin showing epidermal hyperplasia with Mild nuclear +1 immunoreactivity P53 expression in basal and suprabasal keratinocytes ((x200)

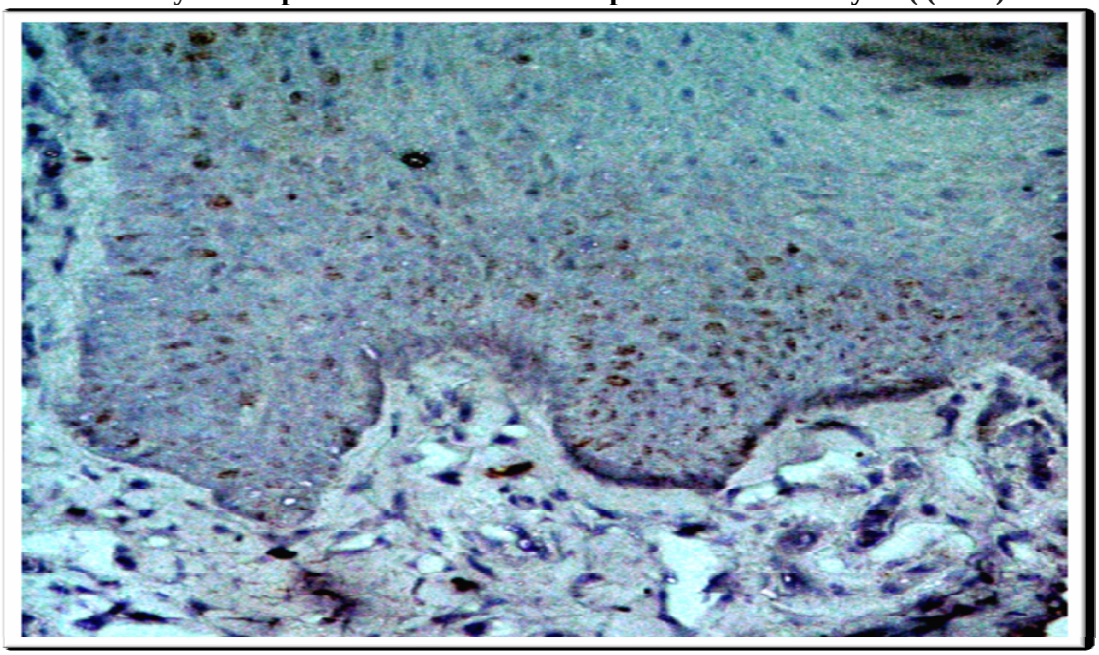


Figure (15): Lesional psoriatic skin showing epidermal hyperplasia with Moderate nuclear +2 immunoreactivity P53 expression in suprabasal keratinocytes (x200)

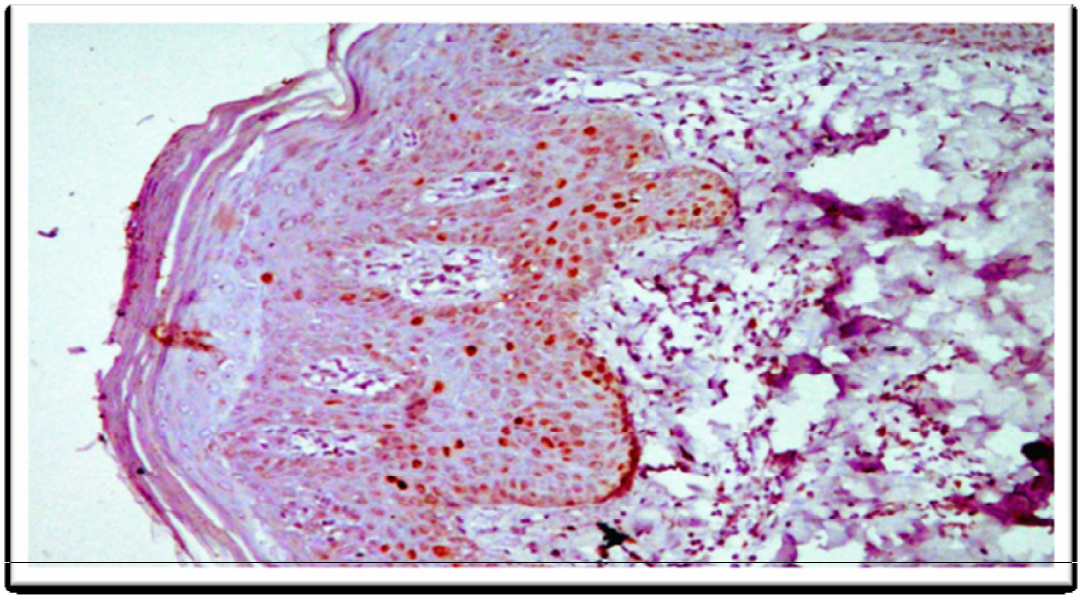


Figure (16): Lesional psoriatic plaque showing epidermal hyperplasia with Strong nuclear +3 immunoreactivity P53 expression in basal and suprabasal keratinocytes (x100)

Discussion

Psoriasis is a benign skin disease characterized by hyperproliferation, abnormal differentiation of keratinocytes, by the presence of inflammatory cell infiltrate in both the dermis and the epidermis and by alterations to the capillaries (Kerkhof ; 2003). Many genes, particularly oncogenes and tumor suppressor genes, could be involved in the disregulation of the cell-cycle (increased cell division), which is probably important in the development of psoriatic lesions (Bishop 1991;Marshall 1991).

Although p53 immunoreactivity has also been found in several inflammatory skin diseases, such as psoriasis, lichen planus, chronic dermatitis and lupus erythematosus (Batinac et al ;

2004) , the results are frequently controversial. Therefore, the present study was undertaken to evaluate the expression of p53 in psoriatic skin. DNA damaging agents induce the activity of this protein, which leads to cell cycle arrest in the G1 or G2 phase, and in the case of ineffective DNA repair apoptosis is initiated (Batinac et al , 2004;Hainaut et al,2000).

The first publication to discuss p53 and psoriasis appeared in 1989, when Tadini et al. reported p53 nuclear expression in psoriatic skin. In contrast to these findings, a few years later Moles et al; 1993 used similar antibodies but did not find p53 positive cells either in lesional or non-lesional skin biopsies taken from psoriatic patients. Later studies performed by Helander et al. 1992, Soini et al.1994 and Hannuksela-Svahn et al. 1999 confirmed the presence of p53 positive keratinocytes in psoriatic epidermis.

P53 positivity was detected in 13 psoriatic specimen (43.3%) in the basal and suprabasal keratinocytes of psoriatic skin, the results of the current study are in accordance with Tadini et al., (1989) which was the first publication to discuss P53 and psoriasis, reported that anti P53 polyclonal antibody (241) was positive in about 20% of the cells in the basal and suprabasal layers of psoriatic skin while polyclonal antibody showed prevalent cytoplasmic positivity and so they argued that P53 is an important marker of active cycling cell in psoriasis and speculated that primary alteration of cell cycle in psoriasis is very similar to those observed in neoplastic transformation and that a control factor may exist in psoriasis that blocks successive progression of the transformative process.

Helander et al., (1992) observed P53 cytoplasmic staining with monoclonal antibodies (421) and (1801) in the basal and supra basal keratinocytes of psoriatic skin.

Hussein et al., (2004) described the presence of P53 positive cells in lesional and

non lesional skin of psoriatic patients with a statistically stronger expression in lesional skin.

Also the results of Soini et al., (1994) agree with our results, where they found aberrant expression of P53 in non-neoplastic inflammatory skin diseases as psoriasis but the percentage of P53 positive cells was lower than seen in malignant tumors. They also found increased proliferation of keratinocytes and they demonstrated a relationship between P53 positivity and the number of ki 67 positive cells and mitosis suggesting that P53 accumulation was enhanced by cell proliferation.

Hannuksela – Svahn et al., (1999) confirmed the presence of P53 positive keratinocytes in psoriatic epidermis and reported that the increase expression of P53 in psoriatic skin is a physiological reaction indicating the attempt to counteract the proliferation and to repair DNA errors, and is most often expressed as an increased number of mitosis.

El-Batawi et al., (2002) found higher P53 expression in lesional than in non lesional psoriatic skin using monoclonal antibody Do7. which is the same as our results. They concluded that the elevated levels of P53 in psoriatic plaques reflect an important role exerted by this tumor suppressor gene on the development of psoriatic plaques.

Kerkhof (2003) demonstrated that P53 is an important marker of active cycling cells in psoriasis and a large body of evidence indicates that the cell cycle time in psoriasis is normal and only increased recruitment of epidermal cells is responsible for the development of psoriatic lesions .

Batinac et al., (2004) examined P53 expression in lesional psoriatic skin, non lesional skin of psoriatic patients and skin samples from neoplastic diseases: basal cell carcinomas and squanous cell carcinomas. P53 immunoreativity was demonstrated in the epidermis of psoriatic skin in the basal and suprabasal layers and only in the basal layer in non lesional skin of psoriatic patients. The difference in P53 expression was however, not significant. In carcinomas (basal cell carcinoma, squamous cell carcinoma) P53 expression was significantly stronger.Our result is consistent with Batinac et al., (2004) who found P53 immunoreactivity was demonstrated in the epidermis of psoriatic skin in the basal and supra basal layers.

On the other hand our data disagree with Moles and associates (1993) who used three different antibodies; polyclonal antibody (CMI) and monoclonal antibodies (Do7) and (1801) that recognize different epitopes for immunohistochemical analysis of P53 protein and they didn't observe nuclear staining in lesional and non lesional psoriatic skin. In addition, they did not detect any mutation in P53 gene using a combination of polymerase chain reaction and single strand conformation polymorphism techniques. Also Magee et al., (1994) found that, staining of P53 was not detected in psoriatic biopsies using anti-P53 Do7 antibodies.

Michel et al., (1996) demonstrated that P53 expression was decreased in lesional psoriatic skin. P53 transcription was clearly induced by antimicrobial substance FK506 (Tacrolimus) treatment, both post translational modification of P53 protein and transcriptional modulation of P53 gene which represent potential targets for the antipsoriatic action of the drug plus down modulation of IL – 8 receptor type A.

Dazard (2000) used immunofluorescence staining of cryosections of 13 psoriatic skin biopsies and could not find P53 positivity in this series of psoriasis cryosections when probed with a panel of specific antibodies.

The wide variation in the results obtained by different works can be attributed to either the difference in the sensitivity of the kits used or to the cross reactivity of anti P53 antibodies with other proteins like keratins (Moles et al., 1993), or due to racial differences.

In the current study P53 expression, either nuclear or cytoplasmic, could not be detected in any of twenty five specimens serving as control. Indicating the significance of P53 study in proliferative disorders, Helander et al., (1993) found a significant cytoplasmic reactivity for P53 was detected in normal cells of control group.

Some theories have been published by Qin et al., (2002) concerning the response of

the keratinocytes to DNA damaging factors, which seems to be different in this instance to that in other cells of human body. Unique mechanisms are essential for the keratinocytes to preserve cutaneous homeostasis and maintain the structural integrity of the skin as a barrier between the organism and the environment.A mutation in the p53 gene has been found in most human tumor types. p53 protein is constitutively expressed in almost all cells, but due to the short span of its half-life it is extremely difficult to detect a "wildtype" p53 in standard immunohistochemistry. The activated or mutated form accumulates in cells and immunohistochemical detection is possible

Our results (which agree with several results and disagree with others) may suggest that the cell cycle is disturbed in psoriatic epidermis but further studies are needed to determine definitively the molecular basis of cell cycle regulation in epidermal cells, and to continue to broaden our knowledge of the pathogenesis of psoriasis.

Summary

The aim of this study was to identify the expression of P53 in psoriatic patients with different clinical types and severity to clarify its role in the pathogenesis of psoriasis and its correlation with the disease severity.

The patients group consisted of 16 males (53.3%) and 14 females (46.6%). Their age ranged between 18 to 60 years with mean $\pm$ SD (36.3 $\pm$ 9.7). The patients were divided into six subgroups according to the clinical types of psoriasis. Five patients (16.6%) with psoriasis vulgaris, 2 of which (40%) show negative intensity of staining of P53 expression and 3 of which (60%) show mild P53 expression. Five patients (16.6%) with guttate psoriaseis all of them (100%) show negative P53 expression. Five patients (16.6%) with psoriasis of palm and sole, 1 of which (20%) shows negative P53 expression and 4 of which (80%) show mild P53 expression. Five patients (16.6%) with Nail psoriasis, 4 of which (80%) show negative P53 expression and 1 of which (20%) shows mild P53 expression. Five patients (16.6%) with flexural psoriasis all of them show negative P53 expression. Five patients (16.6%) with erythrodermic psoriasis; 2 of which (40%) show mild P53 expression and 1 patient (20%) shows moderate P53 expression and 2 patients (40%) show strong P53 expression.

The PASI score of the patients ranged from 3.3 to 67.6 with mean + S.D (22.6+ 22.2) three of the 30 patients (10%) had positive family history, two of which (15.4%) had positive intensity of staining of P53.The expression ranged from mild (10 cases, 76.9%) to moderate (1 case, 7.8%), to strong expression (2 cases, 15.4%).The 25 control group all showed negative P53 expression.

The statistically significant elevation in P53 level with the clinical severity of psoriasis may raise the suspicion that P53 may have a role in determining disease severity and so we argued that P53 is an important marker of active cell cycling in psoriasis.

Conclusion

The results obtained from our study provide further evidence supporting the role of P53 in the pathogenesis of psoriasis as P53 nuclear staining was detected in 13 out of 30 patients (43.3%).

P53 is an important marker of active cycling cell in psoriasis and it reflects an important role exerted by this tumor suppressor gene in the development of psoriasis so P53 is recommended as a routine immunohistochemical marker for psoriasis.

Further studies on larger number of patients and on different clinical types of psoriasis are recommended.

Assessment of P53 levels is recommended after different therapeutic modalities.

There is certainly a hope in the near future that P53 protein comes from only a subject of academic research to being a therapeutic modality adopted in treating inflammatory and neoplastic disorders.

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Protective Effect Of *Nigella Sativa* Seeds Against Dimethylaminoazobenzene (Dab) Induced Liver Carcinogenesis

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ABSTRACT: Liver cancer is one of the most common solid tumors worldwide. Extensive research was carried out to document the powerful properties of *Nigella sativa* (*N. sativa*) as an anti-tumor, bactericide, anticestode, antinematode, anti-inflammatory, analgesic, anti-diabetic and diuretic with many other uses. Little is known about the *Nigella sativa* anti-tumor property in liver cancers, thus our current study was performed to investigate the protective role of *Nigella sativa* in DAB induced liver carcinogenesis. 140 male Albino mice weighing 40-50 gm divided into four groups. Group one was normal control group without treatment of any type. Group two was *Nigella sativa* treated control group. Group three was treated with DAB carcinogen. Group four was treated with both *Nigella sativa* and DAB. Biochemical investigations, flow cytometric analysis, and histopathological examination of the liver tissue were performed for all groups. The results showed that there was a significant change in the DNA content, histomorphology, and antioxidant enzymes in the liver tissues of the DAB treated group. These changes were restored to approximately the normal counterpart with *Nigella sativa* treatment. In addition, treatment with *Nigella sativa* only showed comparable results with control untreated groups on different levels. Collectively, these results give clear evidence that *Nigella sativa* lonely induce no harmful effects on the liver. Moreover, it exerts hepatoprotective effect against liver carcinogens. Antioxidant property is mediated its actions and investigating other underlying mechanisms merits further studies.

Keywords: *Niglla sativa*, Dimethylaminoazobenzene, Liver, antioxidant, Carcinogenesis.

INTRODUCTION

Liver cancer is one of the most common cancers representing a leading cause of cancer related mortality, with an increasing incidence worldwide, being responsible for more than one million deaths annually [1, 2]. Historically, most drugs have been derived from natural products, but there has been a shift away from their use with the increasing predominance of molecular approaches to drug discovery [3]. Among chemotherapeutic or chemopreventive natural sources, *Nigella sativa* seeds come as a good protector being a natural product [4]. *Nigella sativa*, a spicy plant, is cultivated in various parts of the world. The seeds, also known as black cumin or black caraway, are commonly used in the Middle East, Northern Africa and

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India as a condiment in bread and other dishes. The seeds of *Nigella sativa* have been used for medicinal purposes as a natural remedy for a number of illnesses and conditions that include bronchial asthma, rheumatism, hypertension, diabetes, inflammation, cough, headache, eczema, fever and influenza. The seeds or its oil are also used as carminative, diuretic, lactagoge and vermifuge [5, 6].

The effect of *Nigella sativa* has been evaluated in animal studies. There are many reports on its biological activities including immunopotentiation, anti-tumor, anti-inflammatory, analgesic, antihypertensive, anti-diabetic, antiulcerogenic, respiratory stimulation, anti-bacterial, antifungal, anticestode and antinematode effects in the literature [6-8]. *N. sativa* decreases DNA damage and thereby prevents initiation of carcinogenesis in colonic tissue secondary to exposure to toxic agents such as azoxymethane [9]. In fact, sustained delivery of thymoquinone (derived from *N. sativa*) is almost as effective in causing apoptosis of colon cancer cells as sustained delivery of 5-fluorouracil [10]. Similarly, hepatic metastasis from tumors such as mastocytomas is markedly decreased following administration of *N. sativa* [11].

Administration of *N. sativa* oil showed protective effect on the liver of Swiss Albino mice against exposure to radiation [12]. It has been reported that *N. sativa* oil could diminish the CCl4-induced hepatotoxicity, the doxorubicin-induced cardiotoxicity and the harmful effects of some chemicals [5, 13-19]. Abnormal activities of some enzymes are usually indicative of the hepatic cellular injury in animals and humans [20]. Interrelationships between protective effects of the *N. sativa* oil and harmful effects of CCl4 have been investigated, and it has been reported that there is protective and antioxidative role of *N. sativa* oil [5, 14]. Therefore, the present study was designed to investigate the effects of *N. sativa* seeds on activities of functional liver enzymes, antioxidant status and on the liver injury generated by dimethylaminoazobenzene (DAB) in male albino mice through biochemical, histopathological and flow cytometric analyses.

MATERIALS AND METHODS

Animals

A total number of 140 adult male Swiss Albino mice weighing 40-50 g were used in the current study. The animals were maintained on a standard cube diet and free water supply, and were treated for 32 weeks. The animals were divided into four groups each containing 35 rats. The first is the untreated control group. The second is the carcinogen treated group with 0.05% DAB mixed with diet. The third group is the *N. sativa* control group treated only with 5.0 % grinded seeds of *N. sativa* mixed with diet. The fourth group is the treated with 5.0 % *N. sativa* plus 0.05% of DAB mixed with diet. All animals were sacrificed 32 weeks after starting the experiment. The local ethical committee approved the present study.

Blood was collected and sera were separated, divided into aliquots and stored frozen at 70ºC till analysis. Liver specimens were cleared of surrounding fat, cut into small pieces and weighed out. 10 % liver homogenates were prepared in distilled water. Samples were kept cold on crushed ice all times during preparation then kept frozen at -70ºC till analysis. The serum was used to determine alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), total proteins, and serum albumin using standard protocols. Liver tissues specimens of different groups were fixed in 10 % formalin and paraffin embedded for histopathological examination and 5 µm sections were stained with Hematoxylin and Eosin.

Lipid perioxidation and antioxidant enzymes determination

Activity of superoxide dismutase (SOD) was determined by the method of by Winterbourn *et al* [21]. Glutathione Peroxidase (GPx), was determined by method of described by Flohe and Gunzler [22]. Catalase (CAT) was determined by methods of Beers and Sizer [23]. Lipid perioxidation was expressed as level of malondialdehyde (MDA) measured using a thiobarbituric acid assay as reported and described earlier by

Ohkawa *et al* [24]. Protein determinations were assayed by the method of Lowry *et al* [25].

Flow cytometric analysis

A single-cell suspension of tissue specimens was prepared by mechanical disaggregating in RPMI-1640 medium, filtration through a piece of fine nylon mesh, and centrifugation at 1900g for 10 min to remove debris and cell clumps. The cell suspension then was permeabilized with Triton-X 100. Cells were stained at room temperature for 2–3 h in 50 µg/mL propidium iodide and 100 U/mL RNase A in phosphate buffer saline. Samples were analyzed with a FACSCalibur cytometer using Cell-Quest software (Beckton Dickinson, San Jose, CA). Propidium iodide-stained samples were analyzed using doublet discrimination, which permits a distinction to be made between the signals coming from a single nucleus and the ones produced by two or more aggregated nuclei. For the computer analysis, only signals from single nuclei were considered. The DNA histogram derived from each specimen was analyzed using the DNA analysis software. DNA content was derived from the DNA index (DI), DI = G0/G1 peak average channel value in experimental sample DNA histogram divided by G0/G1 peak average channel value in normal cell sample DNA histogram. DNA ploidy was judged according to DI value. DI in diploid cell was 1.0. A diploid DNA histogram was defined as DI=1.0 ± 2CV (coefficient of variation). An aneuploid DNA histogram was defined as DI ≠ 1.0 ±2CV. The results were recorded as normal DNA content (diploid pattern) and abnormal DNA content (aneuploid pattern).

Statistical analysis

The statistical analysis was computed using analysis of variance (ANOVA). The significant mean differences were separated by Duncan's Multiple Range Test. *P* values < 0.05 were considered statistically significant.

RESULTS

The mean body weight of DAB and DAB + N.S treated groups were significantly decreased ($p<0.001$) compared to the normal control group. The mean body weight of the protected group (DAB+N.S) was not significantly increased compared to the DAB treated group as shown in (Figure. 1).

The obtained results indicated that no significant difference in ALT, AST, ALP, total proteins, and serum albumin between *N. sativa* treated group and untreated control group. Serum ALT and AST were slightly elevated in the DAB treated group when compared to the normal control and other groups. The mean level of serum ALP was significantly increased ($P < 0.001$) in the DAB treated group when compared to the normal control group and to the other groups. The mean level of serum ALP was non-significantly elevated in the DAB plus *N. sativa* treated group when compared to the normal control group (Table 1). The mean body weight of DAB alone, and DAB plus *N. sativa* treated groups were significantly decreased ($P < 0.001$) compared to the normal control group. The body weight of the protected group (DAB plus *N. sativa*) was non-significantly increased compared to the DAB treated group.

The results of antioxidant enzymes and MDA analysis indicated that a pronounced elevation of MDA in the DAB treated group, this elevation was significantly different in comparison to the other three groups. On the hand, this elevation of MDA was accompanied with lowering the level of antioxidant enzymes namely SOD, GPx and CAT in the DAB treated group, this decreasing was significantly different in comparison to the other three groups (Table 2). These results clearly indicate that DAB is affecting the liver negatively by increasing the level of perioxidation and meanwhile decreasing the level of antioxidant protecting enzymes. Moreover, since no differences were existed between the other three groups, reflecting that the *N. sativa* treatment have no harmful effect on the liver, and the *N. sativa* treatment in rats previously treated with DAB can effectively maintain the protective enzymatic balance effectively.

In histomorphology analysis, in the control untreated group, the liver was seen strictly with normal architecture of liver cells and bile ducts. On the other hand, the DAB treated group showed 42.9 % with marked

dysplasia of liver cells with cirrhotic liver nodules and the bile ducts showed well differentiated cholangiocarcinoma. 57.1% of this group showed marked bile duct proliferation, early cirrhotic liver changes as well as moderate dysplasia of liver cells. Regarding the *N. sativa* treated group, it showed absolutely normal architecture of liver cells and bile ducts similar to that of the control group. Finally, the DAB plus *N. sativa* treated group, 28.6% of them showed resistant bile duct proliferation with moderate dysplasia of hepatocytes and the other 71.4% showed no cirrhosis, no bile duct proliferation, dysplasia or carcinoma (Figure 2).

In the flow cytometric analysis, the control untreated group showed a single diploid peak. Similarly, the *N. sativa* treated group showed a single diploid peak as well. Regarding the group of rats treated with DAB only, an additional abnormal peak was detected in 84.6% of samples (aneuploid cells) along with the diploid one. 15.4% of samples showed a single diploid peak. Rats treated group with both *N. sativa* and DAB showed a diploid DNA peak in 80% of samples with disappearance of the abnormal aneuploid peak that was detected in the DAB treated group. However, 20% of liver samples showed aneuploidy. On the other hand, the S phase fraction was recorded as 4.6 vs. 5.1 vs. 15.1 vs. 9.8 in untreated control, *N. sativa*, DAB only, and *N. sativa* with DAB treated groups respectively, indicating a significant elevation in DAB treated group versus the others (Figure 3)
.

Table (1): The levels of the ALT, AST, total protein, albumin, and ALP recorded in the different experimental groups.

	ALT	AST	Total protein	Albumin	ALP
Control	8.67± 1.91	98.67 ± 12.87	7 ± 0.25	3.31 ± 0.17	19.92 ± 1.92
DAB	9.93 ± 4.01	108.86 ± 16.63	6.83 ± 0.35	2.72 ± 0.17	28.49± 4.72
N.S	7.92 ± 1.61	100.62 ± 10.56	6.87 ± 0.27	2.97 ± 0.22	19.56± 1.92
N.S. + DAB	8.79± 2.15	103.36 ± 11.90	6.81 ± 0.23	2.78 ± 0.23	23.46 ± 5.76

N.S: *Niglla sativa*; DAB : Dimethylaminoazobenzene.

Table (2): The level of liver MDA and antioxidant enzyme activity (SOD, CAT, and GST-Px) recorded in different groups.

*Significant compared to control

Groups	Liver MDA (nmol/g protein)	Liver CAT (U/mg protein	Liver SOD (U/mg Protein)	Liver GSP-PX (U/mg Protein)
Control	22.6□±□2.4	9.81 ± 0.76	72□±□7	5.97 ± 0.48
DAB	110□±□12.4*	5.96 ± 0.56*	32□±□6*	3.37 ± 0.29*
N.S	24.8□±□7.5	9.06 ±0.9	68□±□8	6.09 ± 0.50
N.S. + DAB	45.4□±□4.2	7.06 ± 0.18	65□±□7	5.90 0.73

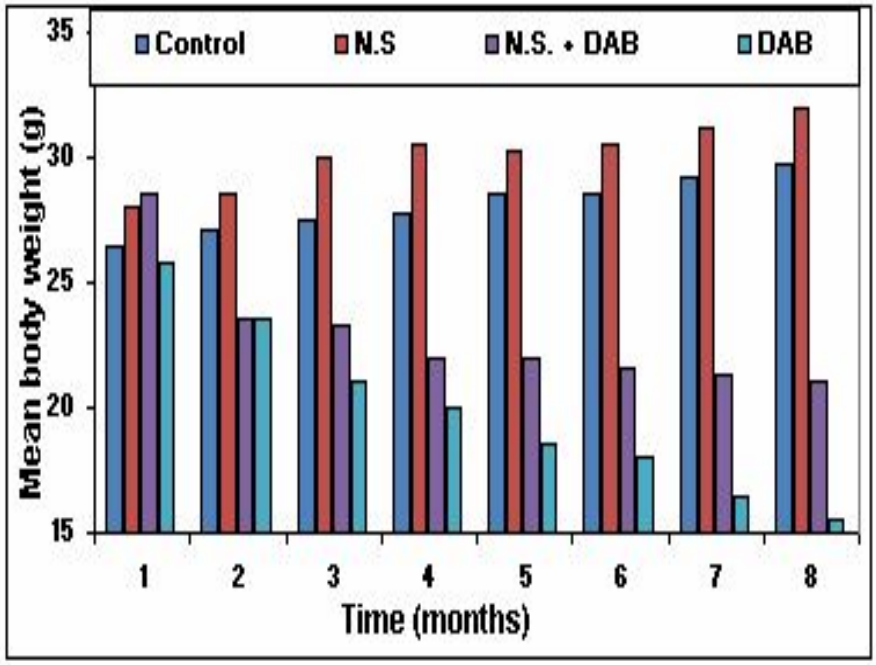

Figure (1): Gradual change in the mean body weight (gm) of control and treated animals

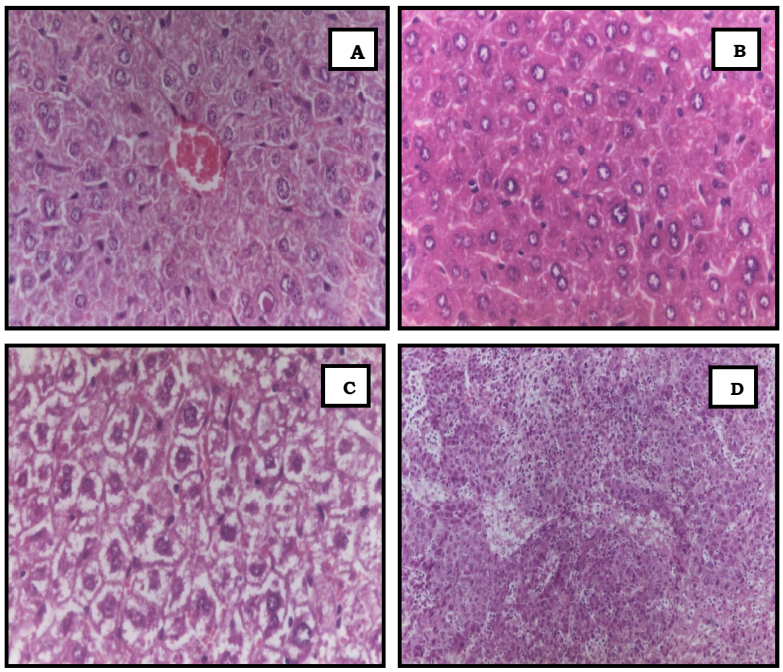

Figure (2): Histomorphology examination of different tissues. (**A**) Liver tissue section of control group stained by hematoxylin and eosin showing normal hepatic architecture[x 400], (**B**) Liver tissue section of treated group with *N. sativa* showing no changes in liver cells and bile ducts [x 400], (**C**) Liver tissue section of animal treated with DAB with *N. sativa* group showing mild dysplasia of hepatic cells [x 400], (**D**) Liver tissue section of treated group with DAB only showing liver cirrhotic nodules and bile duct proliferation [x 400].

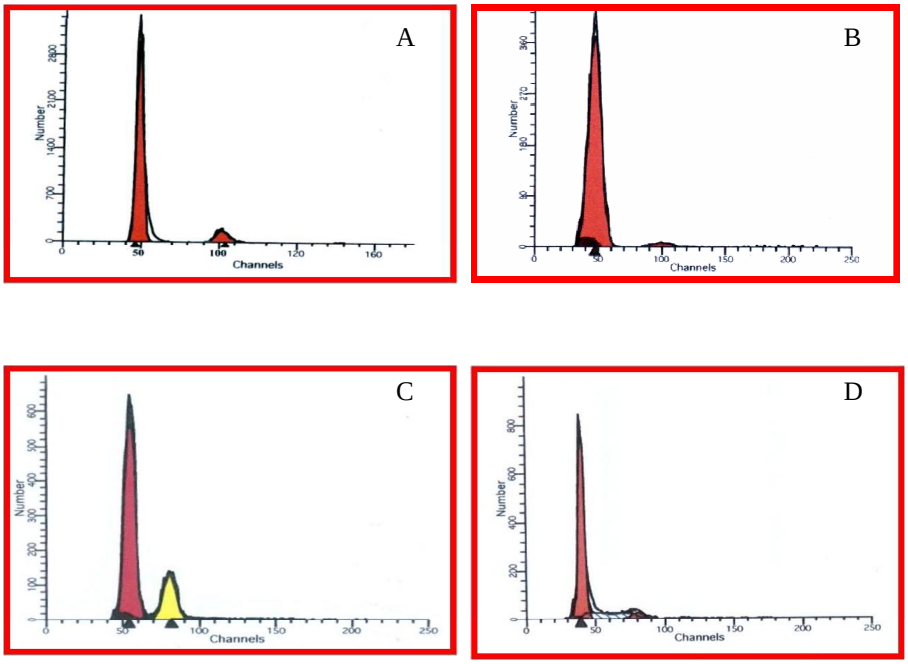

Figure (3): Flow cytometric analysis (A) Control untreated group (one diploid peak); (B) N.S treated group (one diploid peak); (C) DAB treated group (aneuploid peakpattern) and (D) DAB+N.S treated group (one diploid peak with SPF)

DISCUSSION

The liver is the primary target for carcinogen effect of more than two hundred chemicals (including pesticides, food additives, pharmaceuticals, and industrial intermediates) tested in long-term toxicity safety assessment assays [26]. Some of these harmful agents for the liver are carbon tetrachloride (CCl4), N-methyl-N-nitrosourea, 1, 2-dimethylhydrazine, 3'-methyl-4-dimethy -lamino-benzene and ortho-aminoazotoluene. Dimethylaminoazobenzene (DAB) is a well known liver carcinogen induces multiple cellular, molecular, and biochemical changes [27-32].

In the current study, we sought the harmful effect of DAB on the rat liver and the possible ameliorating effect of *N. sativa* treatment in those animals. The results showed significant decrease in body weight in DAB, and DAB plus *N. sativa* treated groups compared to the untreated control group. These results in agreement with others who found weight loss in disturbed liver proliferation activity [27, 33]. There were no significant difference in ALT, AST, total proteins, and albumin in different groups. While Serum alkaline phosphatase showed significant increase in DAB treated group when compared to DAB plus *N. sativa* treated group and to normal control group. There is no significant difference between the DAB plus *N. sativa* group when compared to the normal control group. The present

experimental model using DAB as a hepatocarcinogen induce mainly cholangiocarcinoma and this might explain the normal level of ALT, AST, albumin and total proteins recorded. The present findings are in agreement with prior reports [33, 34].

The flow cytometric results showed that *N. sativa* treated group showed a single diploid peak similar to that of untreated control group, while rats treated with DAB, an additional aneuploid peak was detected. In case of DAB plus *N. sativa* treated group, it was found that most of samples had a diploid DNA peak without the aneuploid one. The present result is in agreement with other reports that showed the aneuploid pattern in majority of cases bearing adenocarcinoma of the extrahepatic bile ducts [34, 35]. Histopathological results *N. sativa* treated group showed normal architecture of liver cells and bile ducts similar to that of untreated control group. In DAB treated group 42.9% of cases showed marked dysplasia of liver cells and cirrhotic liver nodules and the bile ducts showed well differentiated cholangiocarcinoma. Primary carcinoma of the liver can usually be classified as hepatocellular carcinoma (HCC) or cholangiocarcinoma (CC). Approximately 98% of liver cancer is HCC, which originates from the hepatocytes, and approximately 2% is CC, which originates from the epithelium of the intrahepatic bile duct. The carcinogenic mechanism of HCC correlates with chronic liver disease and with persistent HBV or HCV infection, but CC has no relation to viral hepatitis. Combined hepatocellular-cholangiocarcinoma is a rare form of primary liver cancer with features of both hepatocellular and biliary epithelial differentiation [36]. There are many molecular alterations associated with CC including p53 mutation, cyclins, proliferation indices, mucins, CA19-9, and aneuploidy appear to hold significant potential as predictors of outcome in CC [37]. CC is ideally induced using DAB carcinogen in experimental rats [27, 38]. The current study showed in cases treated with DAB plus *N. sativa*, most of the harmful alterations were apparently corrected. These results are comparable with the findings of others [34], and the antitumor action was recorded in addition to hepatobiliary cancers, in different other tumors [6, 11], and this action may strongly relates to the chemical composition and activity of *N. sativa*.

The results of the current study indicated that a pronounced elevation of MDA in the DAB treated group that was accompanied with lowering the levels of antioxidant enzymes investigated. These results clearly indicate that DAB is affecting the liver negatively by increasing the level of perioxidation and meanwhile decreasing the level of antioxidant protecting enzymes. Moreover, since no differences were existed between the other three groups, reflecting that the *N. sativa* treatment have no harmful effect on the liver, and the *N. sativa* combined with DAB treatment can effectively maintain the protective enzymatic balance effectively. The prominent antioxidant activity of *N. sativa* was extensively documented in different experimental situations, including hepatocarcinogenesis [39-43]. It has been shown that both the fixed oil of *N. sativa*, as well as thymoquinone (the main compound of the essential oil), inhibit non-enzymatic lipid perioxidation in liposomes [44]. Using thin-layer chromatography (TLC), it has also been shown that compounds isolated from *N. sativa* (including thymoquinone carvacol, t-anethole and 4-terpineol) have appreciable free radical scavenging properties [5]. These compounds were found in a series of other *in vitro* tests to have antioxidant activity, but no prooxidant properties. The antioxidant action of *N. sativa* may explain its claimed usefulness in folk medicine. This antioxidant property would explain its action against CCl4 hepatotoxicity [14], liver fibrosis and cirrhosis [45], and hepatic damage induced by *Schistosoma mansoni* infection [46].

Collectively, these results give clear evidence that *Nigella sativa* lonely induce no harmful effects on the liver. Moreover, it exerts hepatoprotective effect against hepatobiliary carcinogens. Antioxidant property is mediated its actions and investigating other underlying mechanisms merits further studies.

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Immune Modulation Potentials of Aqueous Extract of *Andrographis paniculata* Leaves in Male Rat

Oyewo, Emmanuel Bukoye and Akanji, Musbau Adewumi

Abstract

The immune modulation potentials of the aqueous extract of *Andrographis paniculata* leaves was investigated. The dry pulverized leaves were extracted with water and lysophilized. Forty male albino rats were randomly picked into four groups. The first group received distilled water, while the other groups were administered daily 250 mg/kg, 500 mg/kg and 1000 mg/kg BW doses for 84 days. Effect of the chronic administration of the extracts on haematological parameters, IL-6, TNF-α, serum levels of bilirubin and uric acid were estimated. The packed cell volume was not significantly changed ($p<0.05$), while haemoglobin and red blood cell were increased significantly ($p<0.05$) only in group four. Dose dependent significant increases ($p<0.05$) were observed in platelet count and erythrocyte sedimentation rate. Mean cell volume was reduced significantly ($p<0.05$), but with no significant differences ($p <0.05$) among the test groups. Mean cell haemoglobin and mean cell haemoglobin concentration showed significant reductions ($p<0.05$) in the group 4 only. The white blood cell and lymphocytes were increased significantly ($p< 0.05$) with group 2 and 3 been statistically equal. Significant reductions ($p< 0.05$) were observed in neutrophil and eosinophils in the group 4 rats only. Monocyte was increased significantly ($p<0.05$) in group 4 only. Dose dependent significant increases ($p<0.05$) were observed in the serum IL – 6 and TNF-α. Total and direct bilirubin decreased significantly ($p<0.05$) in group 2 and 3, while significant increases ($p<0.05$) was shown in group 4. Indirect bilirubin was increased significantly ($p< 0.05$) in group 3 and 4 only. Uric acid was reduced significantly ($p<0.05$) in group 2 and 3, while group 4 showed significant increase ($p<0.05$). The overall result suggested that the chronic consumption of the aqueous extract of *A. paniculata* boosted the immune functions, but the 1000 mg/kg BW dose predisposed to anaemia, possibly multiple myeloma and autoimmunity

Keywords: *Andrographis paniculata,* Immune functions, Chronic consumption, Predisposed, Multiple myeloma

Introduction

Medicinal plants are being used in traditional system of medicine from hundreds of years in many countries of the world (Oubre *et al*., 1970). These plants are not only for primary health care, and not just in rural areas of developing countries, but also in developed countries, where modern medicine are predominantly used (Kamboj, 2000). In recent times, the interest in medicinal plants has increased in a great deal due to its therapeutic properties, which is very useful in

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healing various diseases and the advantage of these plants is being 100% natural (Calixto, 1998). According to the World Health Organization, about 80% of the populations in many third world countries still use traditional medicine for their primary health care, due to poverty and lack of access to modern medicine (De Silva, 1997).

Andrographis paniculata, also known commonly as "King of Bitters," is a member of the plant family, *Acanthaceae.* It is also referred to as the 'bile of earth' since it is one of the most bitter plant that are used in traditional medicine (Coon and Ernst, 2004). The geographical distribution of the plant has led to its traditional use in Ayervedic (Indian), Thai, and Chinese medicine. According to these traditions, andrographis dispels heat (i.e., is antipyretic) and removes toxins, which makes it a good treatment for infectious fever causing diseases. In traditional Chinese medicine, *A. paniculata* is an important cold property herb, used to rid the body of heat and fever. *A. paniculata* extracts inhibited the body's inflammatory mechanism and demonstrated not only anti-microbial abilities, but also were instrumental in killing certain tumor cells. In vitro studies have shown that the flavonoid activities supressed the genetic expression of neutrophiils, an inflammatory agent. *In vitro* studies have also indicated that the active chemical in *A. paniculata*, andrographolide, helps to stop the clumping of blood platelets which is the clotting process that can lead to heart attacks (Amroyan *et al*., 1999). It was also suggested to have a major effect activating the general defense functions of the immune system by stimulating the production of antibodies as well as non-specific immune responses such as increased macrophage phagocytosis, rather than by any direct anti-microbial activity (Amroyan *et al*., 1999).

Immune deficiency is the root of susceptibility to a variety of infections, and it is the basis of the acquired immune deficiency syndrome (AIDS). Impairments of immune function result in variable clinical symptoms. Immunostimulatory activity of *A. paniculata* is evidenced by increased proliferation of lymphocytes and production of interleukin 2 *in vitro*. *A. paniculata* also enhanced the tumor necrosis factor α production and CD marker expression, resulting in increased cytotoxic activity of lymphocytes against cancer cells, which may contribute for its indirect anticancer activity (Rajagopal *et al*., 2003). Laboratory tests conducted in Buffalo, New York, also, demonstrated that *A. paniculata* inhibited the growth of human breast cancer cells at levels similar to the drug tamoxifen (Puri *et al*., 1993).

The consumption of the infused aerial parts of *A. paniculata*, alongside with meals (as blood tonic), is being encouraged, because of the medicinal properties alleged by traditional medical practitioners: as it is believed to enhance immune system functions such as production of white blood cells (scavengers of bacteria and other foreign matter), release of interferon, and increase the activity of the lymph system and to prevent or cure infective and degenerative diseases (Tapsell *et al*., 2006). However, if *A. paniculata* does indeed stimulate the immune system, this could lead to a whole host of potential risks, because the immune system is balanced on a knife edge. An immune system that is too relaxed fails to defend the organism from infections and an immune system that is too active attacks healthy tissues, causing autoimmune diseases. A universal immune booster might cause or exacerbate lupus, Crohn’s disease, asthma, Graves’s disease, Hashimoto’s thyroiditis, multiple sclerosis, and rheumatoid arthritis, among other illnesses. Thus, this study was designed to evaluate the effect of the chronic consumption of *Andrographis paniculata* on the immune system of rats.

Materials and method

Plant material for analysis

The aerial part of *A. paniculata* was collected from the natural habitat around Airport area in Ilorin, Kwara State. The plant was identified by Mr. L. T. Soyewo at Forest Research Institute of Nigeria, Ibadan, Oyo State. A specimen of the plant was kept with voucher number (108453) for future reference. The leaves were rinsed thoroughly in distil water and dried in the shade for 14 days. The dried leaves were ground to fine powder, using a domestic electric grinder and extracted with water at 37^{o}C. The filtrates were pulled together and centrifuged at 2000rpm for 10 minutes. The supernatant was filtered again and lyophilised using a freeze dryer. The yield of the aqueous extract was 16.28%$^{w}/_{w}$. The dried extract was stored in the desiccators and kept in the dark till when needed.

Chemicals

All the chemicals and reagents used in the study were of analytical grades from the Bristish Drug House and Sigma Aldrich.

Quantitative assay kits

IL – 6 and TNF – alpha estimation

IL – 6 and TNF – alpha were quantified with rat enzyme linked immuno-sorbent assay (ELISA) kits produced by Ray-Biotech, Inc. U. S. A.

Bilirubin and uric acid estimation

Bilirubin and uric acid were estimated using reagent kits produced by LABKIT, CHEMELEX, S.A. Pol. Ind. Can Castells. C / Industrial 113, Nau J. 08240 Canovelles – Barcelona.

Laboratory animals

Forty 10–12 weeks old male albino rats of average body weight of 125–140 g were obtained locally from Oyo Town, Oyo State. The rats were housed in animal care facility at the Faculty of Basic Medical Sciences, LAUTECH, Ogbomoso.

Methods

Experimental animals and procedure

The forty male albino rats were randomly grouped into four, comprising of ten rats per group. They were housed in animal care facility at the Faculty of Basic Medical Sciences, LAUTECH, Ogbomoso with 12-hours light/dark cycle. They were fed free standard pellet diet and tap water, and were acclimatized for 10 days before the administration of the aqueous extract of *A. paniculata* was commenced. The cages were cleaned every morning and disinfected 3 days interval. Calculated doses of the plant extracts (mg/kg body weight of rat) were dissolved in distilled water and stored air tight at 4^{0}C. Administration was performed orally at 24 hours interval, using metal cannula attached to a 2ml syringe.

Group 1:Control, received 1.5ml distilled water.

Group 2: Test, received 250 mg/kg body weight of *A. paniculata*

Group 3: Test, received 500 mg/kg body weight of *A. paniculata*

Group 4: Test, received 1000 mg/kg body weight of *A. paniculata*

Administration lasted for 84 days, after which the rats were fasted for 12 hours and sacrificed by anaesthetia, using di-methyl ether. Incision was made quickly in the chest region and the heart was pierced to collect blood into labelled heparinised bottles and labelled non – anticoagulant bottles.

Haematological analysis

The haematological parameters were analysed by the automated haemology analyzer (SYSMEX K2X1: SYSMEX CORPORATION, JAPAN). The mean corposular haemoglobin, mean corpuscular haemoglobin concentration and mean cell volume were calculated.

Serum interleukin – 6 and TNF – α estimation

The serum level of IL-6 and TNF – α was estimated by *in vitro* enzyme linked immunosorbent assay (ELISA) kit, using colourimetric reaction method as instructed in the kit manual with cat # ELR- IL6-001 and ELR - TNF alpha – 001 respectively.

Serum bilirubin estimation

The serum bilirubin levels were estimated by dimethylsulphoxide (DMSO) colourimetric reaction, according to the method as described by (Malloy *et al*., 1937: Kaplan *et al*., 1984).

Uric acid estimation

The serum uric acid levels were estimated by uricase-POD enzymatic colourimetric reaction, according to the method as described by (Fossati *et al*., 1980: Schultz, 1984).

Stastitical analysis

This research work was a completely randomised design (CRD). Results analyses were performed using Prism 3.00 software. The results were expressed as mean ± standard deviation of 3 - 8 replicates where appropriate. Results were subjected to one way analysis of variance (ANOVA) to test the effect of each dose level on the parameter under investigation at 5% degree of freedom. The Duncan Multiple Range Test (DMRT) was conducted for the pair-wise mean comparisons, to determine the significant treatment dose at 5% level of significance. P-value <0.05 was regarded as statistically significant and denoted by alphabets.

Results

The results were presented in tables. The values were expressed as mean ± standard deviation of at least 5 replicates and alphabets were used to depict significantly different ($p<0.05$) mean value.

Physical examinations of rat

Administration of the aqueous extract of *A. paniculata* to albino rats recorded the death of one rat at the 500mg/kg BW dose after 71 days and partial paralysis and/ mortality of three rats at 1000mg/kg BW dose after 50 days. The rats of the 1000mg/kg BW dose group were often less active after administration and consumed more water than the other dose groups. After 32 days of administration, four rats in this group had their fur dropping off, the feaces were watery with the colour faded and the eyes were very red and budged out. However, one of the three paralyzed rats did not die till the end of the experiment. Figure 1 depicts the pictures of the rats in the each group.

Haematological analyses

Results of the haematological estimations were presented in table 1. The administration of the extract had no significant effects ($p < 0.05$) on the PCV of the test groups (table 1). The Hb count and RBC count were increased significantly ($p < 0.05$) for the group 4 rats only. The platelets counts were increased significantly ($p < 0.05$) dose dependently in the test groups. Dose dependent significant increases ($p < 0.05$) were also observed in the ESR of the test groups (table 1).

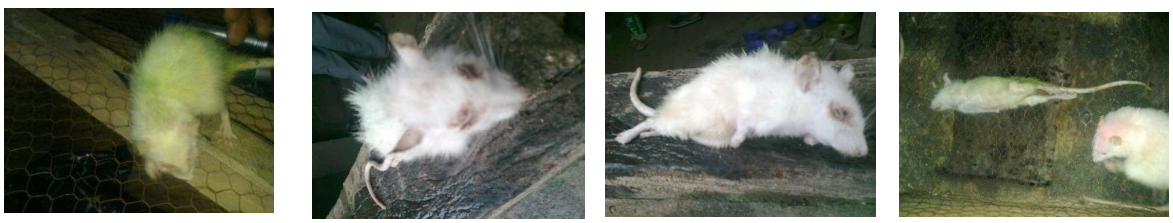

Figure 1: Photograghs of Four Rats in the 1000 mg/kg BW dose group

Table 1: Effect of Aqueous Extract of *A. paniculata* on Haematological Parameters in Male Albino Rats

	Control	250 mg/kg BW	500 mg/kg BW	1000 mg/kg BW
PCV (%)	41.5 ± 1.6432 [a]	39.4 ± 0.5477[a]	40.7 ± 1.2042 [a]	42.4 ± 1.5166 [a]
HBC (g/dl)	12.917 ± 0.7111[a]	13.058 ± 1.3465[a]	13.018 ± 0.9192[a]	11.094 ± 0.6236[b]
RBC (10^{12}) /L	7.2167 ± 0.2316[a]	7.552 ± 0.8081[a]	7.988 ± 0.5608[a]	8.878 ± 0.4006[b]
PLATELET(µl)10^3	334 ± 12.1119[a]	358.8 ± 7.5630[b]	384.2 ± 18.7082[c]	427.6 ± 20.5971[d]
ESR (mm^3/hr)	2.8 ± 0.4472[a]	3.6 ± 0.5477[b]	4.2 ± 0.4472[c]	6.8 ± 0.8367[d]

Red cell indices

The results of the erythrocyte indices (MCV, MCH and MCHC) are depicted in table 2. The MCV was reduced significantly ($p < 0.05$) in the test groups (2, 3, and 4) with no significant difference among the test groups. The MCH and MCHC showed significant reductions ($p < 0.05$) in the group 4 rats only (table 2).

White blood cell count

Results of leukocytes estimations were shown in table 3. The WBC and lymphocytes (L) count were increased significantly ($p < 0.05$) in the test groups with group 2 and 3 been statistically equal. The result of the granular leukocytes (neutrophil, monocytes, basophil and eosinophils) are presented in table 3. Significant reduction and increase ($p < 0.05$) were observed respectively in the neutrophil and monocyte counts in the group 4 rats only. The eosinophils count was reduced significantly ($p < 0.05$) in group 2 and 3 rats, while significant increased ($p < 0.05$) was observed in group 4 rats. Basophils were not detected in group 3 rats, while two rats in group 2 and three rats in group 1 showed basophil count with no tandard deviation. However, basophils were estimated in five rats of group 4.

Serum cytokines

The effect of the extract on serum cytokines (IL - 6 and TNF – α) levels were presented in table 4. Dose dependent significant increases ($p < 0.05$) were observed in the serum levels of IL - 6 and TNF – α in the test groups.

Serum bilirubin and uric acid

The effect on the administration of the aqueous extract on serum bilirubin and uric acid were depicted in table 4. The total bilirubin concentrations was significantly increased ($p<0.05$) in the group 4, while group 2 and 3 showed significant reductions ($p<0.05$). Direct bilirubin concentrations decreased significantly ($p< 0.05$) in group 2 and 3, while significant increase ($p< 0.05$) was shown in group 4 (table 4). The indirect bilirubin levels were increased significantly ($p< 0.05$) in group 3 and 4 only. The uric acid concentrations were reduced significantly ($p< 0.05$) in group 2 and 3, while significant increase ($p < 0.05$) was shown in group 4 (table 4).

Table 2: Effect of Aqueous Extract of *A. paniculata* on Erythrocyte Indices in Male Albino Rats

	Control	250 mg/kg BW	500 mg/kg BW	1000 mg/kg BW
MCV (fL)10^{15}	56.952 ± 3.2970[a]	52.039 ± 2.7083[b]	50.951± 2.3360[b]	47.759 ± 2.5111[b]
MCHC (g/L)	3.112 ± 0.1704[a]	3.322 ± 0.1091[a]	3.294 ± 0.1596[a]	2.617 ± 0.1938[b]

MCH (10^{-12})	0.179 ± 0.0027[a]	0.173 ± 0.0062[a]	0.168 ± 0.0072[a]	0.125 ± 0.0177[b]

Table 3: Effect of Aqueous Extract of *A. paniculata* on White Blood Cells in Male Albino Rats

	Control	250 mg/kg BW	500 mg/kg BW	1000 mg/kg BW
NUETROPHIL (%)	48.4 ± 2.9664[a]	51.8 ± 2.8636 [a]	52.4 ± 2.0736 [a]	32 ± 4.6368 [b]
WBC 10^9 (/l)	3.46 ± 0.6025[a]	4.7 ± 0.3674 [b]	5.5 ± 0.3701 [b]	8.84 ± 0.7668 [c]
LYMPHOCYTE (%)	18 ± 3.1623[a]	24.4 ± 2.0736 [b]	27.1 ± 4.1473 [b]	53.4 ± 5.5946 [c]
EOSINOPHIL (%)	2.5 ±0.5774[a]	1.25 ± 0.5774 [b]	1.33 ± 0.5774 [b]	3.5 ± 0.5774 [c]
BASOPHIL (%)	1.00 ± 0.0	1.00 ± 0.0	0.00 ± 0.0	2.25± 0.5
MONOCYTE (%)	3.6 ± 1.1401[a]	3.8 ± 1.0954[a]	4.2 ± 1.7886[a]	5.6 ± 1.140 [b]

Table 4: Effect of Aqueous Extract of *A. paniculata* on Serum IL-6, TNF-α, Bilirubin and Uric acid in Male Albino Rats

	Control	250 mg/kg BW	500 mg/kg BW	1000 mg/kg BW
IL-6 (pg/ml)	434 ± 42.1901[a]	640 ± 35.3553[b]	752 ± 41.2311[c]	1196 ± 41.5933[d]
TNF –α (pg/ml)	324 ± 15.1658 [a]	450 ± 31.6228[b]	570 ± 46.9042 [c]	910 ± 44.3847[d]
Total Bilirubin (mg/dl)	1.006 ± 0.1137[a]	0.649 ± 0.1154[b]	0.9097± 0.0605[c]	1.345 ± 0.0941[d]
Direct Bilirubin (mg/dl)	0.526 ± 0.1720[a]	0.201 ± 0.0188[b]	0.343 ± 0.0182[c]	0.646 ± 0.0469[d]
Indirect Bilirubin(mg/dl)	0.480 ±0.1134[a]	0.449 ± 0.022[a]	0.566 ± 0.1002[b]	0.699 ± 0.0772[c]
Uric Acid (mg/dl)	4.53 ± 0.4982[a]	3.50 ± 0.2675[b]	3.99 ± 0.1170[c]	6.69 ± 0.1889[d]

Discussion

The volume of red blood cells in whole blood (PCV) was not affected by the chronic administrations of the extract (table 1). However, the extract might have induced anaemia at 1000mg/kg BW dose as observed in the haemoglobin count and increased number of red blood cell (RBC) per liter of whole blood (table 1). The dose dependent increase in the platelet count in the study disconcor with the anaemic capability of the extract (Topley, 1998), and might suggest possible adverse effect on blood metabolism like leukaemia, multiple myeloma, bone marrow infiltration etc (Janeway *et al*., 1997). This observation might be dis-advantageous in vascular endothelial tissues, as increased blood platelets are implicated in atherosclerosis. The increase in the (ESR), which is the rate at which RBC sediment on their own weight per unit time, supported the salient anaemic capability of the extract (table 1). The reduction in the mean cell volume signifies that the size of the RBC were reduced, indicating microcytic anaemia due to either iron deficiency anaemia and/ anaemia of chronic disease (Topley, 1998). Reduction of the mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) at the 1000mg/kg BW dose (table 2), supported the possibility of the extract predisposing the consumer to iron deficiency anaemia and or, microcytic hypochromic anaemia (Vasudevan and Sreekumari, 2000).

The aqueous extract of *A. paniculata* could have erythrocytes building capability due to the level of iron, as previously reported by Oyewo *et al*. (2010). However, the presence of lead may suggest possible toxicity to the liver because no safe level of lead in blood has been established. More so, the

incorporation of iron into protoporphyrin ring in haem biosynthesis is inhibited by lead, which could result to acquired porphyria, hypochromic-microcytic anaemia and hyperuricemia (Jeremy *et al*., 2001: Champe *et al*., 2005).

The extract elucidated a boost of the white blood cell and lymphocyte (table 3). White blood cells (leukocytes) are involved in fighting infection and clearing off damaged or dead cells and tissues in body (Jeremy *et al*., 2001). However, excessive count of white blood cells (WBC) are implicated in arthritis, trauma, uraemia, leukamia, myeloproliferative disorder, haemorrhage, myocardial infaction, inflammation, tissue necrosis, stenuous excercise and acute infections (Vasudevan and Sreekumari, 2000).

Reduced WBC count are reported in viral, bacteria and parasitic infections, hypersplenism, anaemias, bone marrow infitration and anaphylatic shock (Tracey and Cerami, 1994). The sharp increase in WBC and lymphocyte counts (lymphocytosis) at the 1000 mg/kg BW dose requires carefully examination, as the increase may be due to any of the aforementioned conditions. Reduction in the blood level of neutrophils (neutropenia) at the 1000 mg/kg BW dose (table 3) may indicate leukaemia, neoplasia, tissue damage, anaphylaxis, malignant disease, splenomelay, megaloblastic anaemia etc (Topley, 1998). The decrease in the blood eosinophil level at the the 250 mg/kg BW and 500 mg/kg BW doses may reduce the possibilities of parasitic infections, malignancies, lymphomas, connective tissue diseases and allergic reactions, while increase in eosinophil blood level at the 1000 mg/kg BW dose could predispose to these conditions. The blood basophil levels observed in this study, though not tested statistically, might indicate that the 1000 mg/kg BW dose of the extract could predispose to myeloproliferative disorders and some allergies. The increase observed in the blood monoctye level (table 3) in the 1000 mg/kg BW dose support the immune boosting capabilities of the aqueous extract of *A. paniculata,* but may suggest possible chronic myelomonocytic leukaemia (Tracey and Cerami, 1994).

The dose dependent increases in the serum IL-6 and TNF-α in the study strongly indicate the immune modulation potential of the aqueous extract of *A. paniculata*. IL-6 and and TNF-α are important cytokines involved in the differentiation and proliferation of the immune cells (Janeway *et al*., 2001). IL-6 is secreted by monocytes /macrophages, fibroblasts, endothelial cells, keratinocytes, mast cells, T cells and many tumor cell lines to stimulate immune response to trauma, skin burns or other tissue damage leading to inflammation and fever. It is one of the mediators that are released very early in an injury process (Murtaugh *et al*., 1996). IL-6 stimulates the acute phase reaction, which enhances the innate immune system and protects against tissue damage (Abbas *et al*., 1997). It increases the synthesis of the two major acute phase proteins, C-reactive protein (CRD), which increases the rate of phagocytosis of bacteria and serum amyloid A (SSA), by regulating the transcription of their genes. IL-6 has major effects on haematopoiesis, thrombopoiesis and appears to be a growth factor of malignant cells (Roitt, 1991). Increased levels of IL-6 in blood correlate with increased synthesis of fibrinogen, erythrocyte sedimentation rate, secretion of glucocorticoids, and the activation of the complement, the B cells and the clotting cascade, and decreased blood albumin (Castell *et al*., 1989: Kushner *et al*., 1990: Abbas *et al*., 1997).

IL-6 has been reported to have positive and negative actions on metabolic responses in liver, adipose tissue, and skeletal muscle. It is relevant to many disease processes, in which elevated serum or plasma levels may occur in different conditions including such as

diabetes (Kristiansen and Mandrup-Poulsen, 2005), systemic lupus erythematosus, sepsis, lymphoid malignances, multiple myeloma, autoimmune diseases, lymphomas, AIDS, alcoholic liver disease, Alzheimer disease and in organ infections or transplant rejection (Abbas *et al*., 1997: Tackey *et al*., 2004). IL-6 stimulates energy mobilization in the muscles and fat tissues, which leads to increased body temperature. IL-6 in particular is thought to worsen the symptoms of autoimmune diseases and fibromyalgia (Febbraio and Pedersen, 2005). Interleukin-6 has been found to act as a growth factor in several tumors and some viruses also use IL-6 to replicate. Interleukin-6 also causes calcium to be released from bone, promoting osteoporosis (Tracey and Cerami, 1994). Moreso, elevated levels of IL-6 may be associated with an increased risk of atherosclerosis, heart attack and stroke (Dubiński and Zdrojewicz, 2007), prostate cancer (Smith *et al*., 2001), and rheumatoid arthritis (Nishimoto, 2006). Advanced or metastatic cancer patients have higher levels of IL-6 in their blood, hence, there is interest in developing anti-IL-6 agents as therapy against many of these diseases (Barton, 2005: Smolen and Maini, 2006). Finally, if acute – phase response, inflammation and fever are not controlled they become detrimental, leading to sepsis and shock.

TNF-α is produced by activated macrophages and other cell types including T and B cells, NK cells, LAK cells, astrocytes, endothelial cells, smooth muscle cells and some tumor cells (Aggarwal and Reddy, 1994). The primary role of TNF-α is in the regulation of immune cells. TNF- α is also able to induce apoptotic cell death, to induce inflammation, and to inhibit tumorigenesis and viral replication. Most organs of the body appear to be affected by TNF-α, which has both growth stimulating and inhibiting properties. TNF- α induces neutrophil proliferation during inflammation and also induces neutrophil apoptosis upon binding. TNF- α aids in maintaining homeostasis by regulating the body's circadian rhythms. It also promotes the replacement of injured cells and senescent tissues by stimulating fibroblast and stimulates immune response to bacterial, fungal, viral and parasitic infections, as well as, necrotic cells (Janeway, 1997). TNF- α also induces the secretion of acute phase protein and increases the vascular permeability to recruit macrophages, neutrophils and CD_8 T killer cells. It causes the blood clotting so as to contain the infection. TNF- α is known to cause the apoptosis of tumor cells, but it over expression could promote the growth of the tumor cells (Locksley *et al*., 2001).

Over-production of TNF-α, however, has been implicated as playing a role in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders. Dysregulation and, in particular, overproduction of TNF-α have been implicated in a variety of human diseases, as well as cancer (Locksley *et al*., 2001). High blood levels of TNF- α correlate with increased risk of mortality (Rink and Kirchner, 1996). TNF-α is believed to mediate pathogenic shock (sepsis) and tissue injury associated with endotoxemia. High blood levels of TNF-α increased the risk of heart disease by 79 percent and of heart failure by 121 percent (Cesari *et al*., 2003). When TNF-α production increases to the extent that it escapes the local infection, or when the infection enters the blood stream, sepsis occurs. Sepsis shock results to fever, falling blood pressure, myocardial suppression, dehydration, acute renal failure and finally, respiratory arrest. Excess production of TNF-α in the body reduces blood volume, albumin level and neutrophil (systemic edema), body organs fail and death ensues. Prolong over-production of TNF- α leads to the loss of the anti-tumor activiy, due to polymerization of TNF- α molecules and the shedding of the

receptors. The symptoms of prolonged TNF-α production are anorexia, net catabolism (lipid and protein), weight loss, hepatosplenomegaly, insulin resistance, endothelial activation, anaemia, illness such as cancer and AIDS (Tracey and Cerami, 1994). Recent studies have demonstrated that IL-6 and TNF-α are stronger predictors of cardiovascular disease than C-reactive protein. In the health, aging and body composition study, people with the highest IL-6 levels were two to five times more likely to have a heart attack, stroke or other cardiovascular episode than those with the lowest levels (Cesari *et al*., 2003). Prolong over productions of IL-6 and TNF- α, lead to sepsis shock and organ failure, and ultimately, paralysis and death (Dubiński and Zdrojewicz, 2007).

The significant reduction in the serum total bilirubin at 250mg/kg BW dose (table 4) could be adduced to enhanced haem metabolism and bile excretion, while the 500mg/kg BW dose also enhanced haem metabolism and bile excretion, but not significantly. However, the dose of 1000mg/kg BW possibly caused imbalance in haem metabolism and excretion, which resulted to the increase in the serum total bilirubin level. The dose dependent significant increase in serum total bilirubin among the test groups may be due to increased anaemia or increased red blood cell degradation, biliary stricture (benign or malignant) and chronic liver disease (Vasudevan and Sreekumari, 2000: Champe *et al*., 2005). Increased total bilirubin causes jaundice, which is characterized by the yellowing of eyes and the skin (Friedman *et al*., 1996). The significant reduction in the serum direct bilirubin concentrations at 250mg/kg BW and 500mg/kg BW suggested the possibility of the aqueous extract of *A. paniculata* to protect and enhance hepatocytes and biliary functions at the doses. However, the increase in the serum direct bilirubin at the 1000mg/kg BW dose, suggested possible biliary obstruction and hepatotoxicity, which could lead to obstructive jaundice, haemolytic jaundice and hepatocellular jaundice (Vasudevan and Sreekumari, 2000: Champe *et al*., 2005).

The non significant reduction in the serum indirect bilirubin at 250mg/kg BW dose may suggest that the aqueous extract of *A. paniculata* did not cause significant anaemia , possibly haemolytic anaemia (Vasudevan and Sreekumari, 2000). Dose of 500mg/kg BW indicated possible predisposition to haemolytic anaemia that was strongly supported by the 1000mg/kg BW dose. Thus, it was logical to infer that the 1000mg/kg BW dose could predispose to obstructive jaundice (increased serum direct bilirubin), haemolytic jaundice (increased serum indirect bilirubin and hepatocellular jaundice (increased serum indirect and direct bilirubin) (Vasudevan and Sreekumari, 2000: Jeremy *et al*., 2001). However, the 500mg/kg BW dose could possibly predispose to haemolytic jaundice only. More so, the possibilities of obstructive and or hepatocellular jaundice in the 1000mg/kg BW dose group was strongly supported by the faded colouration of the rat feaces during administration of the aqueous extract of *A. paniculata*. This was probably due to hepatocellular damage or the obstruction of the secretion of conjugated bilirubin into bile by the liver, which led to the diffusion (leakage) of conjugated bilirubin into the serum. The disruption in the secretion of conjugated bilirubin into bile would lead to the decrease in the enterohepatic circulation and more urobilinogen is excreted in the urine (responsible for the dark colouration), while less conjugated bilirubin is secreted into the gut during lipid metabolism. Thus, the level of urobilin and stercobilin in the feaces are decreased, so the feaces would appear clay coloured (Vasudevan and Sreekumari, 2000: Champe *et al*., 2005).

The significant decrease in the serum

uric acid concentration (hyporuricemia) observed at 250mg/kg BW and 500mg/kg BW doses of the aqueous extract of *A. paniculata* implied that there were reduced tissues degradation (turn over due to trauma and high rate of catabolism as in starvation), low possibilties of growing malignant tissues (lymphomas, polycethemia, leukaemia), renal injury or failure, lead poisoning and gout (Jeremy *et al*., 2001: Champe *et al*., 2005). Low levels of uric acid in the blood are seen much less commonly than high levels and are seldom considered cause for concern (Aringer and Graessler, 2008). Interestingly, uric acid has been shown to be a very strong reducing agent *in vivo*. It has comparable antioxidant capabilities as ascorbic acid and is often used as a biomarker for oxidative stress (Glantzounis *et al*., 2005: Baillie *et al*., 2007).

In humans, over half the antioxidant capacity of blood plasma comes from uric acid. However, like other strong reducing substances such as ascorbate, uric acid can also act as a pro-oxidant particularly at elevated levels (Proctor, 1970: Cutler, 1984). Thus, it is unclear whether elevated levels of uric acid in diseases associated with oxidative stress such as stroke and atherosclerosis are a protective response or a primary cause (Becker, 1993). Therefore, the increase in serum uric acid concentration between the 250mg/kg BW and 500mg/kg BW doses (table 4) may suggest a boost in the antioxidant system. On the other hand, a high uric acid level may not cause problems in all cases, but some people develop gout, kidney stones or kidney failure. A high uric acid level may appear prior to the development of high blood pressure, heart disease or chronic kidney disease, but it's often unclear whether high uric acid level is a direct cause or merely an early warning sign of these conditions (Heinig and Johnson, 2006). The significant increase in the serum uric acid levels at the 1000 mg/kg BW dose may suggest possible risk of increased tissue degradation (turn over due to trauma and high rate of catabolism as in starvation), high possibilties of growing malignant tissues (lymphomas, polycethemia, luekaemia), renal injury or failure, lead poisoning and gout (Jeremy et al., 2001: Champe *et al*., 2005). More so, the probabilty of developing other aforementioned disease conditions associated with hyperuricemia would be increased at the dose level. From the overall findings of this study, it was logical to make the submission that the administration of the aqueous extract of A. paniculata at the 1000 mg/kg BW possibly predisposed to multiple myeloma due to the marked increases in IL-6 and TNF- α levels, whose symptoms includes: anaemia, bone pain and tenderness, hyperuricaemia, nerve damage, neutropenia, hypoalbuminaemia, thrombocytopenia and renal diseases (Vasudevan and Sreekumari, 2000).

Conclusion

The aqueous extract of *A. paniculata* presented a boost in the immune system of rat at all the dose levels studied. The boost of the immune system recorded was attributed to the increase in the serum TNF- α and IL-6 levels. However, the possibilities of anaemia indicated by the reduced haemoglobin count, increased platelet count and erythrocyte sedimentation rate, reduced erythrocyte indices and neutrophil count of the test groups, especially at the 1000mg/kg BW dose was strongly supported by the increased serum levels of IL-6 and TNF- α, which also suggested the autoimmune capability of the chronic consumption of the extract. To explain clearly the metabolic effects of increased serum levels of IL-6 and TNF-α, studies is currently on to evaluate the effect of the chronic administration of the aqueous extract of *A. paniculata* on glucose utilization, lipid and protein profiles in male albino rats.

Acknowledgement

We acknowledge the assistance of Messrs Adedeji Laurence L. and Adekunle A. S. of Biochemistry Department, Ladoke Akintola University Technology, Ogbomoso to the success of this work. The technical contribution of Messrs Akinyinka and Salisu of Chemical Pathology Unit of University of Ilorin Teaching Hospital, is also appreciated.

Disclosure Statement

“No competing financial interests exist."

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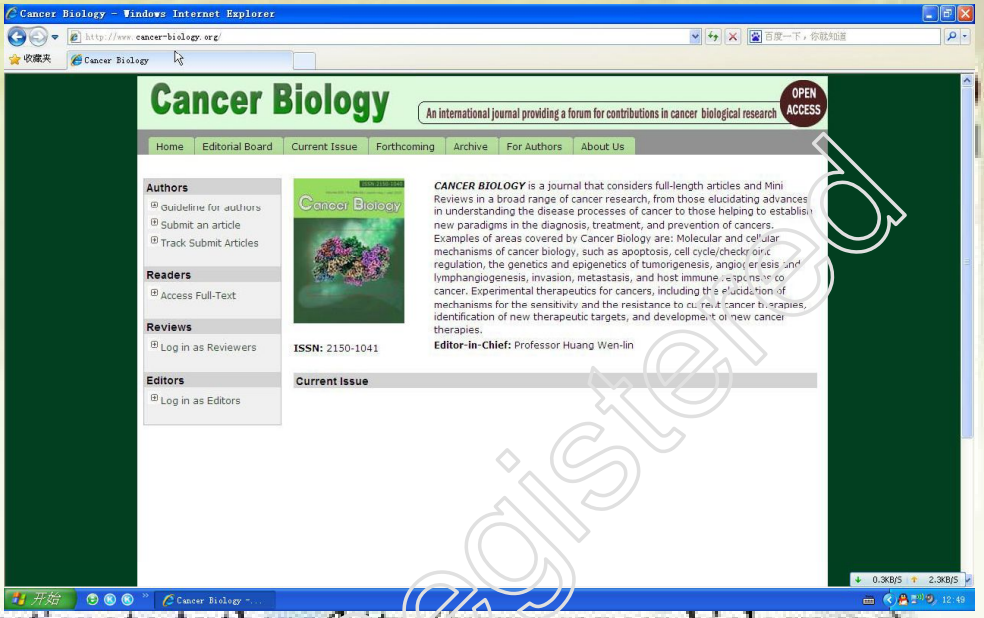

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