International Journal of Cancer Research 1 (1): 1-4, 2005
ISSN 1811-9727

Sentinel Lymph Node Biopsy in Intraductal Carcinoma of the Breast with Microinvasion

Mattia Intra, Jack Antonio Diaz Brito, Javier Soteldo, Serife Simsek and Umberto Veronesi
Department of Surgery, Breast Unit, European Institute of Oncology, Milan, Italy

Abstract: Ductal carcinoma *in situ* with microinvasion (DCISM) is a separate clinical and pathological entity, distinct from pure ductal carcinoma *in situ* (DCIS), with a low but well-known metastatic potential. Due to the low rate of axillary metastases in DCISM, there is controversy regarding the indication for complete axillary dissection (CAD) to stage the axilla. Sentinel lymph node biopsy (SNLB) could be routinely proposed to accurately stage the axilla avoiding the morbidity of a CAD. From March 1996 to December 2002, out of 4602 SLNBs performed for invasive carcinoma of the breast, 41 patients with DCISM in the definitive diagnosis were selected. Metastasis in the SLN were detected in 4 of 41 (9.7%) patients. Two of the 4 patients had only micrometastasis in the SLN. In three of these patients, the SLN was the only positive node after CAD. SLN biopsy should be considered as a standard procedure in DCISM patients. Complete AD may not be mandatory if only the SLN contains micrometastatic disease.

Key words: Breast cancer, ductal carcinoma *in situ*, microinvasion, sentinel lymph node, metastasis

INTRODUCTION

Ductal carcinoma *in situ* with microinvasion (DCISM) represents less than 1% of all breast cancers[1]. Although microinvasion is a property of infiltrating disease, the potential for lymphatic metastatic spread is not obvious and the subsequent optimal surgical approach to the axilla is controversial[2-4].

The aim of this study was to determine the prevalence of SLN metastasis in a series of patients with DCISM of the breast, to determine the clinical usefulness of the SLNB in these patients and, finally, to identify if patients at higher risk of dissemination to the axillary lymph node exist.

MATERIALS AND METHODS

Between March 1996 and December 2002, 4602 patients with clinically node-negative breast carcinoma underwent SLNB at the European Institute of Oncology in Milan, Italy and were prospectively included into a database. To be offered SLNB, the patients had to have cytologically or histologically verified breast carcinoma 3 cm or less in size (measured clinically and/or by imaging techniques) and clinically uninvolved axillary lymph nodes.

Among these patients, 41 patients (aged between 29 and 67 years, average 35.6) affected by DCISM, were included in the current investigation.

Lymphoscintigraphy: Lymphatic mapping was performed using a radiocolloid technique as previously described[5]. Briefly, 5-10 MBq of ^{99m} Tc-labeled colloidal particles of human albumin size range 20-80 nm, (Nanocol; Nycomed Amersham-Sorin, Saluggia-VC, Italy) in 0.2 ml of isotonic sodium chloride solution were injected close to the tumor subdermally or peritumorally, the day before surgery or the same day. Lymphoscintigraphy was then carried out 5-30 min post injection and repeated after 3 h if no SLNs were evident in early images. The skin projection of the lymph node was then marked and used as a landmark when beginning the operation. If the primary tumor was non-palpable we performed a new technique that we called ROLL (Radioguided Occult Lesion Localization) to localize the tumor using Tc-99m macroaggregates. For SLN identification the same procedure described above was performed on the skin projection of the occult lesions[6]. In case of diffuse microcalcifications in which total mastectomy was indicated, the lymphoscintigraphy was performed using a single subdermal periareolar injection of the radiotracer (Nanocol, Nycomed Amersham-Sorin, Saluggia-VC, Italy)[7].

Surgery: SLN biopsy took place 4-20 h after injection of radiolabeled albumin. A gamma ray-detecting probe (Neoprobe 2000, Ethicon, Inc, Somerville, NY) was employed to locate the radioactive lymph node and facilitate its removal. All the nodes uptaking the

Corresponding Author: Mattia Intra, Via Ripamonti, 435, 20141 Milano, Italy
Tel: +39 02 57489725 Fax: +39 02 57489780 E-mail: mattia.intra@ieo.it

radiotracer were classified as sentinel nodes and were removed for histopathologic examination.

Histopathologic examination: The surgically removed breast lesions were thoroughly sampled for histopathologic examination. In case of microcalcifications, the specimens were sliced and subjected to x-ray examination to ensure complete sampling of all the microcalcification-containing tissue. Specimens without calcifications were extensively sampled taking at least one block/cm of the lesion. Samples from the surrounding tissue were also examined and in case of mastectomy, the areola- nipple complex was also evaluated histologically[8-11].

The SLNs were bisected fresh along the major axis if larger than 5 mm and fixed in 10% formalin for 6-8 h, before being embedded in paraffin. Lymph nodes less than 5 mm were embedded uncut. Fifteen pairs of paraffin-embedded sections, 4 µm thick, were cut at 50 µm intervals. If residual tissue was left, additional pairs of sections were cut at 100 µm intervals until the lymph node was entirely sectioned. One section of each pair was stained with hematoxylin and eosin (H and E).

RESULTS AND DISCUSSION

In 20 patients the clinical presentation was a palpable breast mass, 3 patients had diffuse microcalcifications, 14 patients had a single cluster of microcalcifications and 3 patients had non palpable mammographic or ultrasonographic abnormalities (Table 1). One patient's presentation was unknown. Out of 41 patients, 31 (75.6%) were treated with wide resection and 10 (24.4%) with mastectomy. Fifty-one SLNs were identified and examined: 25 patients had one SLN, 10 had two SLNs and 2 had three SLNs.

Pathologic findings: SLN metastasis were detected in 4 of the 41 patients (9.7%). Two of the 4 patients with a positive SLN had only micrometastasis (<2 mm). The SLNs were the only affected nodes in 3 patients who underwent subsequent CAD. In the other macrometastatic SLN patient, 4 metastatic I Berg level lymph nodes were found. No immunohistology was needed for the detection of metastasis (Table 1).

DCIS with microinvasion (DCISM) is a separate clinico-pathologic entity, distinct from pure DCIS without microinvasion[12-16].

In our series, using the TNM classification and following the criteria of Rosen and Obermann[8] for the definition of the primary tumor, we found 41 DCISM (0.9%) of 4602 consecutive breast cancer submitted to breast surgery and SLNB, with a 9.7% incidence of metastatic lymph nodes.

Table 1: Main characteristics of 41 DCISM patients evaluated

	SLN positive N=4(9.7%)	SLN negative N=37(90.3%)
Clinical presentation*		
Diffuse microcalcifications	0 (0%)	3 (8.3%)
Palpable mass	3 (75%)	17 (47.3%)
Non-palpable opacity	0 (0%)	3 (8.3%)
Cluster of microcalcifications	1 (25%)	13 (36.1%)
Histology		
Ductal	3 (75%)	34 (91.2%)
Lobular	1 (25%)	2 (5.4%)
Other	0 (0%)	1 (2.6%)
Tumor grade°		
G1	2 (50%)	5 (13.9)
G2	2 (50%)	12 (33.3%)
G3	0 (0%)	19 (52.8%)
Hormonal receptor status°		
Oestrogen receptor +	2 (50%)	13 (36.1%)
Oestrogen receptor -	2 (50%)	23 (63.9%)
Progesterone receptor +	2 (50%)	21 (58.3%)
Progesterone receptor -	2 (50%)	15 (41.7%)
Proliferative rate (Ki67)°		
# 20%	2 (50%)	19 (52.8%)
> 20%	2 (50%)	17 (47.2%)
Perivascular invasion		
Present	0 (0%)	0 (0%)
Multifocal		
No	3 (75%)	33 (89.2%)
Yes	1 (25%)	4 (10.8%)
Comedo-DCIS associated	0 (0%)	10 (27.2%)

*In 1 patient the clinical presentation was unknown. ° In 1 patients Tumor grade, Hormonal receptor status and proliferative rate were undetectable. DCISM: Ductal carcinoma *in situ* with microinvasion. SLN: Sentinel Lymph Node

Table 2: Axillary involvement in DCISM patients treated by axillary dissection

References	Years	N° patients	Axillary metastasis
Wong[5]	1990	41	0 (0%)
Rosner[18]	1991	36	1 (2.7%)
Silver and Tavassoli[6]	1998	38	0 (0%)
Jimenez[19]	1998	69	5 (7.2%)
Le Bouëdec[20]	1999	60	3 (5%)
Padmore[21]	2000	11	0 (0%)
Wasserberg[15]	2002	57	3 (5.3%)

DCISM: Ductal carcinoma *in situ* with microinvasion

The current prevailing view is that microinvasive carcinoma carries a very low risk of associated axillary nodal metastatic disease, which is similar to extensive high grade DCIS in several series and has a comparable favorable prognosis with a low risk of subsequent development of metastatic disease and death[15]. For this reason, some authors[13,16-19] have advocated no CAD for DCISM (Table 2). The very low rate of axillary involvement in all reported cases could be due to the fact that all these patients were treated with the standard CAD in which only three to six H and E stained sections per lymph node are cut at 100-500 µm intervals and examined. This standard technique is not able to detect all axillary micrometastasis as SLNB does and could underestimate the axillary involvement rate of DCISM[20-24]. Immunocytochemical staining for cytokeratins or other epithelial markers may be helpful for reducing the risk of missing micrometastatic foci but did not increase the rate

of axillary metastasis when they were compared to H and E stained serial multi sections technique. In only 4% of the positive cases the H and E findings were questionable and cytokeratin immunostaining on the adjacent section was useful for confirming the presence of malignancy.

The techniques of lymphatic mapping and SLN biopsy have also been applied to cases of pure DCIS and DCISM in some series. Previous study demonstrated that, due to the low prevalence of metastatic involvement, in pure DCIS completely excised by radical surgery, SLN biopsy can be avoided. It may only be considered in cases of DCIS with a higher risk of harboring an invasive component at definitive histology (large solid tumors or diffuse or pluricentric microcalcifications), especially undergoing mastectomy, in which a successive SLN biopsy can no longer be performed[25]. Present results confirm previous results: four patients (9.7%) of 41 affected by DCISM had metastatic sentinel nodes[26-27]. In two cases, there were micrometastasis. In three cases, no further positive lymph nodes were found after CAD. In one patient with macrometastatic SLN, four further positive axillary lymph nodes were found.

Due to the low number of positive SLN patients and the subsequent imbalance of the two groups, it is impossible to perform any kind of comparison between the SLN positive and negative DCISM patients (Table 1). Anyway, with the only exception of the clinical presentation, we did not observe any difference between the biological characteristics of the two groups. Only the clinical presentation of the neoplasm seems to be important to predict the risk of SLN metastasis. In three of 4 patients with metastatic SLN a large breast mass was palpable while in non-metastatic SLNs group only 47% of patients had palpable mass. Anyway, any kind of conclusion in this direction is impossible.

In conclusion, due to the significant rate of axillary metastasis in DCISM breast cancer, SLN biopsy should be considered a standard procedure in all these patients. SLNB can detect lymph node micrometastasis and accurately stage the axilla avoiding morbidity of a CAD. Complete AD may not be mandatory if only the SLN has micrometastatic disease. Informed consent and a careful discussion with the patients are very important in this decision.

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International Journal of Cancer Research 1 (1): 5-9, 2005
ISSN 1811-9727

HER-2/neu Gene Amplification in Prostate Cancer from Egyptian Patients by Fluorescence *in situ* Hybridization

Magdy Sayed Aly
Department of Biology, Faculty of Science, Cairo University (Beni-Suef Branch), Cairo, Egypt

Abstract: Prostate cancer is the most frequent malignancy and the second leading cause of cancer deaths among males in the Western world. A study of 40 cases of prostate cancer is conducted in an attempt to identify prognostic biomarkers that can distinguish aggressive cases that must be treated immediately. HER-2/neu oncogene amplification was initially studied because amplification of this gene has been reported in many other types of cancer. In this study, HER-2 gene amplification was assessed by fluorescence *in situ* hybridization (FISH) using a HER-2/neu gene probe with a chromosome 17 centromere control probe. The study was performed on formalin-fixed, paraffin-embedded tissues. FISH successfully analyzed all cases. Only 5 out of 40 (12.5%) were found to be amplified. This frequency was lower than the frequency of amplification found in other in other cancers studied. The level of amplification observed was correlated with the pathological grade. Our data indicate that HER-2/neu gene amplification status can be determined by FISH on archival prostate cancer specimens, significantly correlates with high tumor grade and is more frequently encountered in tumors with advanced pathological stage. Also, FISH is a sensitive technique for detection of abnormalities in the HER-2/neu gene and further studies should be undertaken to determine whether a FISH-based HER-2/neu detection method may prove of importance in the prediction of prognosis and planning of therapy in prostate cancer patients.

Key words: Fish, fluorescence *in situ* hybridization, HER-2neu, oncogene amplification, prostate cancer

INTRODUCTION

Prostate Cancer (PC) is the most commonly diagnosed cancer in men in the Western world and the second leading cause of male cancer death[1,2]. Risk factors for PC include age, race, country of origin and familial history.

An identifying number of recurring chromosomal abnormalities, identified in various tumors during the last years, have resulted in major advances in the understanding of the pathogenesis of malignant transformation. Although prostate cancer is the most common malignant disease in men in Western countries, knowledge about cytogenetic data are still limited as compared to other solid tumors. This is due to the failure to obtain a sufficient number of dividing cancer cells *in vitro*, the low mitotic index of the original tumor in most cases and the overgrowth by normal cells of the cancer cells in culture[3].

No molecular markers are currently available to accurately predict clinical outcome or to discriminate between aggressive cancers that will grow quickly and kill and those that will grow slowly for several years without serious ill effects.

Oncogene amplifications are manifestations of genetic instability, which has been implicated in the pathogenesis of many cancers. It is a characteristic of cancer cells that allows an increased production of specific proteins used for the acquisition and maintenance of the malignant phenotype. For example, it is known that approximately 25-30% of breast and ovarian carcinomas have amplification of the HER-2/neu gene, although the prognostic value of HER-2/neu amplification or over expression in other cancers has been controversial[4-9]. The HER-2neu oncogene, which is located on chromosome 17q21, encodes a transmembrane tyrosine kinase receptor family[10]. The receptor's role in oncogenesis can be derived from its action on cellular cascades involving proliferation and differentiation of epithelial cells.

The present study exploits the availability of the fluorescence *in situ* hybridization (FISH) technology for studying the amplification of HER-2/neu oncogene in prostate cancer cases to determine a subgroup of patients

Corresponding Author: Dr. Magdy Sayed Aly, Department of Biological Sciences, Faculty of Science, King Khalid University, P.O. Box 9004 Abha, Saudi Arabia
Tel: +966507531152 Fax: +96672290165 E-mail: magdyaly@yahoo.com

who are at risk for rapid disease progression can be identified. Our ultimate goal is to define prognostic biomarkers that can predict clinical outcome. Biomarkers that are able to discriminate the fast progressing cancers from the slow growing cancers are most urgently needed not only to save on the cost of the therapy, but also to preserve the quality of life and to avoid unnecessary morbidity associated with therapy[11,12]. A routinely available method to successfully identified abnormal DNA markers will be important in modern oncology practice.

MATERIALS AND METHODS

The primary population for study was prostate cancer patients who underwent radical prostatectomy at National Cancer Institute, Cairo, Egypt. Formalin-a consulting pathologist identified fixed paraffin-embedded tissue blocks from 40 Egyptian patients. One case of these patients was classified as stage T_1, 15 cases as T_2, 23 were classified as stage T_3 and one case as T_4. The lymph node status on all patients was none (No). Histopathological evaluation was performed using the Gleason System[13]. Gene amplification was determined by FISH using the CEP-17/HER-2/neu dual-probe FISH protocol (Vysis Corp, Downer's Grove, IL). Briefly, formalin fixed, paraffin embedded tissue sections (4 µm thick) were deparaffinized in xylene, dehydrated in 100% ethanol and air dried. After a brief wash in phosphate buffer saline, the slides were dehydrated serially and air-dried in the dark. Hybridization with the Spectrum Green HER-2/neu DNA probe and Spectrum Orange CEP-17 DNA probe was carried out according to the manufacture's instructions, using codenaturation block, overnight hybridization and rapid wash procedures. Nuclei were counterstained with DAPI 1 g mLG^1 in Vectashield mounting media (Vector Laboratories, Burlingame, CA). Details of the FISH protocol have been discussed extensively elsewhere[14-17]. The procedure for oncogene amplification has been previously described[16]. Briefly, the raw data on the number of HER-2/neu gene copy number and chromosome 17 centromeric numbers were entered on a two-way table. The average copy numbers for both were calculated separately using the marginal totals in the table. Division can then calculate the amplification ratio. Slides with amplification ratios of less than 1.5 will be classified as “nonamplified”. Slides with amplification ratios = 1.5 will be classified as “amplified”. Ratios between 1.5 and 2.0 will be considered as “low amplification”. Ratios between 2.1 and 4.0 will be considered as "moderate amplification”. Ratios exceeding 4.0 will consider as “high amplification”.

RESULTS

Forty Egyptian patients with prostate cancer were included in this study. The histopathologic grade and clinical stage of the tumors studied are summarized in Table 1. One case of these patients was classified as stage T_1, 15 cases as T_2, 23 were classified as stage T_3 and one case as T_4. The median age of the patients was 63 years (range 47-73 years). Gleason scores were available for all patients and were 2-4 in 4 patient, 5-7 in 29 patients and 8-10 in 7 patients. No lymph node involvement or metastasis at the time of transurethral prostatectomy was detected.

Table 1: Results of FISH analysis of HER-2/neu oncogene amplification

Case #	Age	Stage	Gleason score	Amplification ratio
1	66	T_3	7	3.00
2	48	T_3	6	1.20
3	73	T_3	7	1.43
4	67	T_3	7	2.86
5	71	T_4	9	4.15
6	66	T_3	6	1.27
7	65	T_2	7	1.38
8	66	T_3	7	1.36
9	67	T_3	6	1.40
10	68	T_2	7	1.60
11	63	T_2	3	1.12
12	61	T_3	5	1.43
13	71	T_3	7	1.30
14	58	T_3	5	1.46
15	70	T_2	8	1.31
16	70	T_3	7	1.44
17	67	T_3	7	1.29
18	71	T_1	7	0.90
19	66	T_2	8	1.30
20	56	T_2	4	1.30
21	71	T_3	5	1.39
22	79	T_3	9	1.45
23	70	T_3	8	1.45
24	62	T_3	5	1.44
25	67	T_3	7	1.40
26	68	T_2	6	1.31
27	63	T_2	3	1.30
28	61	T_3	5	1.42
29	71	T_3	7	1.39
30	58	T_3	8	1.27
31	70	T_2	5	1.12
32	70	T_3	7	1.20
33	54	T_3	5	1.20
34	59	T_3	8	1.29
35	61	T_2	6	1.29
36	70	T_2	6	1.25
37	47	T_2	5	1.31
38	50	T_2	7	1.75
39	65	T_2	4	1.22
40	55	T_2	5	1.10

Table 2: Relationship of HER-2/neu amplification to pathological grade

HER-2/neu amplification	Totals[a]	AMP[b]	Dipolid[c]
T_1	1	0	1
T_2	15	2	13
T_3	23	2	21
T_4	1	1	0

a: n=40, b: Amplification, c: Normal cell

HER-2/neu gene amplification, as determined by FISH, was performed on all cases studied. Among all the cases successfully analyzed by FISH, 5 (12.5%) showed HER-2/neu oncogene amplification and 35 (87.5%) showed no amplification (Table 1). The level of amplification observed was correlated with the pathological grade. The only one case with grade T_4 showed high-level amplification, 2 cases with grade T_3 showed moderate-level amplification and 2 cases with grade T_2 showed low-level amplification, according to previously defined criteria (Table 2).

DISCUSSION

The present study was conducted to determine whether HER-2/neu oncogene can serve as biomarker to predict patient prognosis for prostate cancer.

The HER-2/neu gene codes for a transmembrane 185 kDa class I tyrosine kinase receptor protein related to the epidermal growth factor receptor, the amplification or overexpression of which is implicated in the pathogenesis of many human cancers[18-20]. Currently available data suggest that there is an increased HER-2/neu expression in prostate cancer by Western and Southern blotting[21-23]. Moreover, the study of HER-2/neu and other oncogene amplifications using immunohistochemistry has not yielded unequivocal results. FISH technique may prove to be a superior technique to overcome this problem[24].

Our study is not in agreement to prior reports of frequent HER-2/neu gene amplification in patients with prostate carcinoma. Ross *et al*.[25, 26] previously reported that HER-2 overexpression in patients with prostate carcinoma by IHC was 29% and that gene amplification was observed in 41% of patients. Those authors also found that both overexpression and amplification were correlated with tumor grade. An earlier study by Kuhn *et al*.[27] reported from their studies that HER-2 overexpression is not a prognostic marker for prostate cancer. In contrast to breast cancer, FISH detects HER-2/neu amplification in a substantial proportion of prostate cancers that do not overexpress HER-2/neu by IHC[28]. Thus, further investigation is needed to clarify these apparently discordant results[29].

In the present study we evaluated 40 patient specimens for HER-2/neu oncogene amplification. All cases were successful. As noted above, the frequency of HER-2/neu gene amplification found in the present study is less than other previous studies of some of the other types of cancer (Table 1). Mark *et al*.[16] found 11% HER-2/neu amplification in rhabdomyosarcoma. In another study of 40 cases of stage I to stage IV breast cancer, the overall frequency of HER-2/neu oncogene amplification was 22.5%. Furthermore, the highest frequency of oncogene amplification was found to be associated with the highest stages. The highest level of amplification was also found in the higher stages. Based on these results, it is evident that a comparable frequency and level of HER-2/neu amplification in prostate cancer was found[30]. Moreover, Mark *et al*.[17] demonstrated that the frequency of gene amplification is much lower and the level of amplification is less intense in their study of prostate cancer.

Oxley *et al*.[31] concluded that the increased Her-2/neu oncogene copy number appears to be rare in clinically localized prostatic adenocarcinoma and is related to chromosome 17 polysomy rather than true amplification. As a result, it would not be a useful biomarker for identifying those patients who will have recurrences after radical prostatectomy.

It has been shown that HER-2 expression in archival tumor specimens in patients with prostate carcinoma that has been progressed to hormone-refractory prostate carcinoma is infrequent[32].

It was also demonstrated by Skacel *et al*.[33] that low-level amplification of HER-2/neu in prostate cancer was found in 26% of cases (3 to 5 signals per nucleus, corrected for chromosome 17 aneusomy). The presence of HER-2/neu amplification was associated with high tumor volume (>2.0 cm (3), $p = 0.004$). Also Edwards *et al*.[34] showed low levels of HER2 gene amplification (8, 7/89) using FISH technique and HER-2 protein expression (12%, 11/89) using IHC.

Nevertheless, high levels of Her-2/neu expression in androgen-independent tissue have not been observed universally. Morris *et al*.[35] reported that approximately 40% of 10 androgen-independent samples over-expressed Her-2 (compared with 14% of androgen-dependent samples) in their screening program for Phase II trastuzumab and paciltaxel trial. Reese *et al*.[36] using the Tab 250 antibody, reported an even lower rate of Her-2 positivity in patients with androgen-independent disease: Only 3 of 22 patients had intermediate to high expression levels (2+ or 3+). Other reports also suggest that HER2 protein over-expression and gene amplification are relatively uncommon in androgen-independent prostate cancer[37].

In conclusion, HER-2/neu gene amplification status can be determined by FISH on archival prostate cancer specimens, significantly correlates with high tumor grade and is more frequently encountered in tumors with advanced pathological stage. Also, FISH is more sensitive than IHC for detection of abnormalities in the HER-2/neu gene and further studies should be undertaken to determine whether a FISH-based HER-2/neu detection

method may prove of importance in the prediction of prognosis and planning of therapy in prostate cancer patients. Exploration of other biomarkers in prostate cancer using FISH is warranted.

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International Journal of Cancer Research, 1 (1): 10-15, 2005
ISSN 1811-9727

Measurement of an Optical Parameters: Absorption Scattering and Auto-florescence of Skin *in vitro*

S. Firdous, M. Ikram, [1]M. Nawaz and [1]M. Aslam
Laser Laboratory, Department of Physics and Applied Mathematics,
Pakistan Institute of Engineering and Applied Sciences, Nilore, Islamabad, Pakistan
[1]Applied Physics Division, Pinstech, Post office Nilore, Islamabad, Pakistan

Abstract: In this study the chicken breast skin tissues were illuminated with collimated radiation of 400-700 nm Nd-YAG pumped dye laser and measured skin optical properties for dry and hydrated sample *in vitro*. Total reflectance and transmitted intensities were recorded by which scattering, absorption and anisotropic factors of the sample obtained using double integrating sphere setup. The *in vitro* optical parameters are higher then *in vivo* measurements. Our *in vitro* results are in agreements with other data available in literature. Hydration of skin is found to influence its scattering properties. Dry sample scatter less then hydrated sample. Skin auto-florescence spectra were acquired under different excitation wavelength, it shows difference between normal and malignant tissues.

Key words: Optical imaging, light transmittance, absorption, scattering, auto-florescence, *in vitro*, double integrating sphere

INTRODUCTION

Skin is a turbid medium. It constitutes a protective barrier against physical damage of underlying tissue invasion of hazardous chemical and bacterial substances, through the activity of its sweet glands and blood vessels. It helps to maintain the body at constant temperature. The skin is consist on outer protection layer epidermis an inner layer the dermis and stratum corneum. Upper layer of epidermis, consists of dead cells, the dermis is composed of vascularised fibrous connective tissues the subcutaneous tissue, located underneath the skin, in primarily composed of adipose tissue (fat), skin is exposed to the environment, at which incident radiation is refracted. The refractive index[1] is different from the adjacent structure, which may scatter the penetrating radiation. Scattering and refraction at an irregular interface increase the average path length of the penetrating radiation and thereby need the depth of penetration. The understanding of light transport in tissue is an active and an important research area because of its potential applications in medical diagnostic, therapeutic and surgical procedures[2]. Skin is the region most quantitatively studied. It is representation of other organs and it is the first boundary crossed for many therapeutic uses of light. The effects of optical radiation on human skin ranging in scale from molecular to organ are of considerable attention. To understand various models for radiation transfer through the skin, one has to have knowledge of scattering, absorption, refraction, reflection and transmission of skin. Once such a model is established it become possible to compute from only a few measurements the intensity profile of radiation penetrating the skin. Kubelka-Munk model has been applied several times to skin[3]. The direct measurements of angular distribution of radiation transmitted through skin were performed by Lu *et al.*[4] at high wavelength scattering by stratum corneum and epidermis has measured directly by Du *et al.*[5] he placed skin layer in front of monocharometer, in this way he measured direct transmittance intensity. It is concluded that epidermal scattering is large for irregular refractive and reflective interfaces.

In this study we applied the model *in vitro* for skin tissue coefficients and concentrate on the epidermis, stratum cornium and dermis. The data we obtain for skin optics, will be useful for the study of phenomena such as erythema, carcinogenesis and pigmentation, which are almost certainly influenced by skin transmission.

Many values for the absorption and scattering coefficients determined *in vitro* and *in vivo* have been published[6,7]. The relative reflectance as measured at the

Corresponding Author: S. Firdous, Laser Laboratory, Department of Physics and Applied Mathematics,
Pakistan Institute of Engineering and Applied Sciences, Nilore, Islamabad, Pakistan
E-mail: sfirdous@pieas.edu.pk

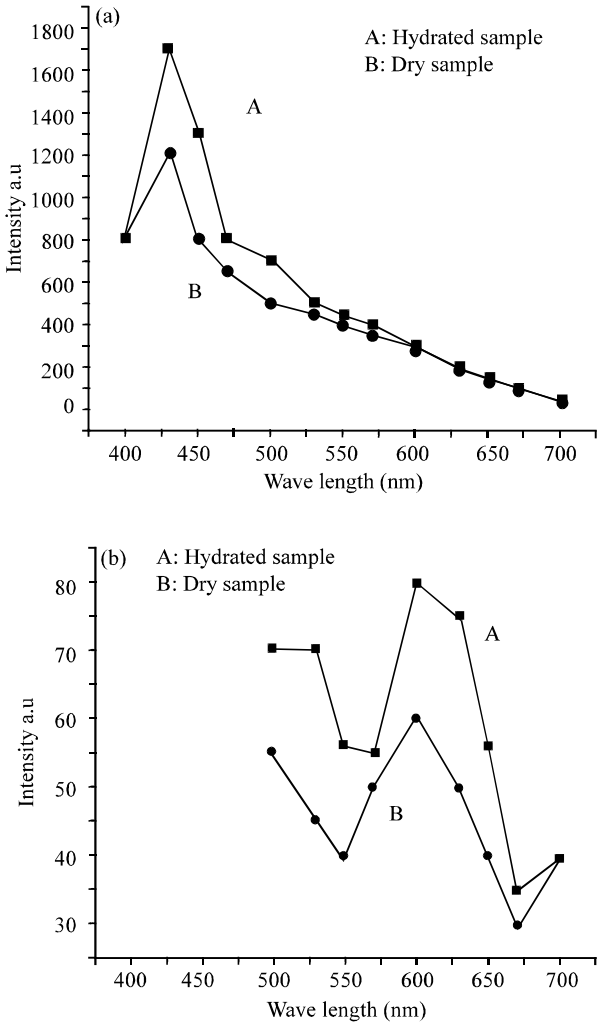

Fig. 3: Auto-fluorescence of chicken breast skin tissue for hydrated and dry sample. (a) Non heated. (b) Heated

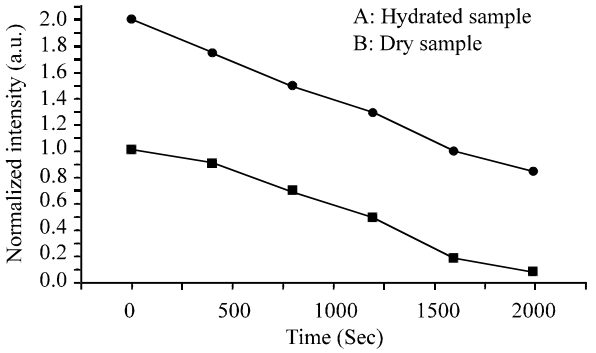

Fig. 4: Transmitted intensity verses time behavior of hydrated and dry sample

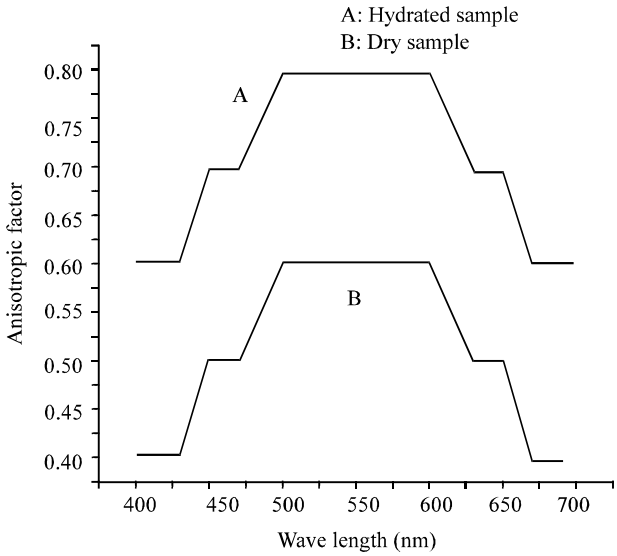

Fig. 5: Anisotropic factor "g" as a function of wavelength

scattering at 470 nm. On comparing our result to Jacques *et al*.[17] it become clear that in our sample scattering is less. The decrease in scattering and absorption, due to surface or volume scattering reduction cannot be separated, volume scattering in Fig 2b, present in hydrated sample disappear in dry sample. When the optics of full thickness skin are being modeled, dermis scattering is the most important, as the penetrating radiation is quickly diffused. It is still just an approximation of the true solution. A more extensive theory would require for sophisticated numerical model for computation, which cannot be performed very accurately because of the large biological variations.

The experimental found values for μ_a and μ_s are influenced slightly by melanin content of epidermis[18].

Therefore small absorption coefficient cannot be determined accurately, as the sum of R_{total} and T_{total} is close to 01. Moreover loss of light within the sample holder may contribute to an increase in the absorption coefficient of tissue. Monte Carlo simulation in good agreement with those measured by Song *et al*.[19].

Absorption measurement evidences a large variation from tissue to tissue of the absorption coefficient value ranging from <02 to 20 cm^{1}. This fact is easily explainable due to different blood contents, which still remain in the sliced samples. Different samples have variety in absorption coefficients. However integrating sphere in cavity measurements make it possible to estimate reasonably the absorption coefficient of sample with a large scattering coefficient. Scattering measurements shows non-isotropic pattern. The anisotropic g factor value are some what higher then those reported (0.81),

under the hypothesis that cell and there internal structure can be approximated by spherical particles and that the main contribution to scattering is due to the cell envelope. The difference between the measured values of ua and us is due to measuring, calculation techniques and sample handling etc.

The measurements from integrating sphere is not straight forwarded, several studies on accuracy of measuring with integrating sphere shows that errors may be due to that the light which leaves the sample re-enter the tissue sample after reflectance. Measurements of total transmission depend on reflectance properties. One of the reasons of difference in results taken by Jacques *et al*,[17] by our calculations, is that they heated the sample which changes the scattering properties of the tissue. Scattering is almost doubles when the sample is heated. The value of g started to decrease slightly at less then 500 nm and greater then 650 nm (Fig. 5). Reflective index was taken as 1.4 for turbid medium and 1 for surrounding. We consider the rectangular shape of detector[20] different values of μ_a and μ_s can be used to obtain the same intensity ratios at detector.

In Fig. 3 auto-fluorescence data is taken from 400-700 nm excitation wavelength and present the curve average of our several experiments. The data is similar to whole skin *in vitro* auto-florescence and maximum at 420 nm for dry and hydrated sample is maximum at 450 nm wavelength. The results are similar to the results obtained by Kobayashi *et al*.[21]. Florescence responsible for emission in this range may be proteins and NADH.

Although proteins absorption region located at shorter wavelength. NADH considerably presents in upper epidermis layer. In Fig.3 (b) we noted small maximum at 510 nm region. For the whole skin *in vitro* and 442 nm excitation of skin strong peak was observed at 632 nm, this peak is distributed to natural porphyrins (excited band covered short line) in the sample. The spectra of dry skin is taken in dark at room temp, during first 24 h fluorescent intensity decreases by 07% and after four days to 70%, but at shorter wavelength rate of degradation is higher.

The optical properties verses time behavior of the skin tissues is shown in Fig. 4. It concluded that intensity should be measured for skin *in vitro* not later then one day after sampling. The moisture effect should be considered for intensity measurements in Fig. 5 the anisotropic factor g is shown as the function of wavelength and maximum at 500 to 650 nm.

CONCLUSION

The optical property of skin is very important for the light dosimetry. For the exact tissue parameters measurement and spectroscopic study, boundary condition and side way photon loses are very important.

The method of our *in vitro* optical parameter measurement is not suitable to *in vivo*, but it gives important information for skin tissue modeling. The experimental data and the investigations were performed at 400-700 nm (visible and near IR). It is reasonable to assume that the scattering coefficient is slightly dependent on wavelength in the visible range. This is not true for absorption coefficient, since tissue blood content and specific absorbing pigments play the main role in determining tissue characteristic. This fact should be kept in consideration while performing the evaluation of light flux distribution in depth. More efforts, therefore be made to determined more precisely the absorption and scattering coefficients and how they are related to blood flow contents.

Analysis of auto-florescence spectra of skin sample is suggested as a mean for skin status diagnostic and monitoring. The optical property of chicken skin tissue is different for different tissues. The variation in coefficients most likely was due to biological variation, preparations of sample and prolonged freezing time, which leads to cell rapture.

ACKNOWLEDGMENT

This project is partially supported through Ph.D merit scholarship of Ministry of Science and Technology, Government of Pakistan.

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International Journal of Cancer Research 1 (1): 16-20, 2005
ISSN 1811-9727

Expression of Cyclin D1 in Tamoxifen Resistant Subline of Human Breast Cancer T47D Cells

[1]Shamileh Fouladdel, [2]Zahra Motahari and [1,2]Ebrahim Azizi
[1]Molecular Research Laboratory, Department of Pharmacology and Toxicology
[2]National Center of Excellence of Toxicology, Faculty of Pharmacy,
Tehran University of Medical Sciences, Tehran, Iran

Abstract: In the present study, the protein expression of cyclin D1 was determined in human breast cancer T47D cells and its TAM-resistant subline (T47D/TAMR-6 cells). The resistant subline was established *in vitro* following stepwise increase in TAM concentration in the culture medium of parent cells. Immunocytochemical method was used to determine the expression of cyclin D1 in both parent and resistant cells. The slow growing T47D/TAMR-6 cells showed lower level of expression of cyclin D1 protein compare to parent T47D cells. Expression of cyclin D1 protein was significantly decreased in both cell types following exposure to TAM (1 μM). Therefore, it can be suggested that TAM can regulate the expression of cyclin D1 protein that affects the cell proliferation. In conclusion, determination of protein expression of cyclin D1 in tumor samples of patients would be helpful in tumor prognosis and prediction of outcome of chemo-hormonal therapy of breast cancer.

Key words: Cyclin D1, Tamoxifen resistance, breast cancer, immunocytochemistry

INTRODUCTION

The cyclin-dependent kinases are a family of serine/threonine kinases that play an important role in controlling progression through the cell cycle[1,2]. Dysregulation of the cell cycle control system is an almost uniform aberration in tumorigenesis[3]. The cyclins encode regulatory subunits of the kinases which phosphorylate specific proteins, including the retinoblastoma (pRB) protein, to promote transition through specific cell cycle checkpoints[2,4]. Cyclin D1 plays a crucial role in G1/S phase of cell cycle progression in fibroblasts and mammary epithelial cell proliferation[5,6]. Overexpression of cyclin D1 is found in 1/3 of human breast cancers that correlates with poor prognosis[7]. Several different oncogenic signals including mutations of the Ras and "-catenin pathway induce cyclin D1 expression[2,8]. Mammary-targeted expression of cyclin D1 is sufficient for the induction of mammary adenocarcinoma and cyclin D1$^{-/-}$ mice are resistant to ErbB2-induced tumorigenesis[9,10]. In addition to binding cyclin-dependent kinases 4 and 6 (cdk4 and cdk6) and pRB, cyclin D1 forms physical associations with P/CAF (p300/CBP-associated factor), Myb, MyoD and the cyclin D1 myb-like binding protein (DMP1)[11,14]. Cyclin D1 binds to the estrogen receptor alpha (ER") and enhances reporter gene activity but inhibits androgen receptor reporter gene activity[14-16]. The *in vivo* or genetic evidence indicating a requirement for cyclin D1 in nuclear receptor function needs to be more elucidated.

Anti-estrogens, especially tamoxifen, are the drugs of choice for the treatment of all stages of breast cancer[17-20]. Tamoxifen as a classical anti-estrogen and its new congeners as well as compounds known as Specific Estrogen Receptor Modulators (SERMs) are regarded as competitive inhibitors of estrogen receptors[17,21,22]. As results of hormonal therapy, gene transcription, DNA synthesis and cellular proliferation are lowered or prevented[23,24]. Unfortunately, in the systemic treatment of patients with advanced breast cancer, the occurrence of drug resistance in patients is a major problem. Therefore, despite the tremendous therapeutic and commercial success of tamoxifen since its introduction, intrinsic or acquired resistance in almost all patients during prolonged treatment needs to think of the important question that is which therapeutic regimen could be most effective after the occurrence of tamoxifen resistance[25, 26]. Answer to this important question requires

Corresponding Author: Ebrahim Azizi, Ph.D., Director of Molecular Research Laboratory, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran
Tel/Fax: +9821 6959100 E-mail: aziziebr@sina.tums.ac.ir

D1 was significantly less in both cell types and in particular in parent cells [Fig. 3C (1+) and D (1+)]. In all conditions a heterogeneous pattern of immunostaining was seen in both cell types.

DISCUSSION

It is known that cyclins and CDKs play important roles in cell proliferation which can affect patients' tumors prognosis[3-7]. On the other hand, the benefits of hormonal therapy of breast cancer have been widely accepted. Nevertheless, almost half of the tumors are less likely to respond effectively to anti-hormonal treatment. Similarly, some of the patients do not respond to chemotherapy[17-20]. In the systemic treatment of patients with advanced breast cancer, the occurrence of drug resistance in some patients is a major problem. The reasons for tamoxifen resistance have not yet been fully elucidated, but several possibilities have been suggested. These include metabolic changes during transport to and into the tumor cells, disturbance of binding to the estrogen receptor or during receptor dimerization, modifications in the binding process of the homodimer/estrogen complex to DNA and/or modulation in the activation of the transcription cascade[27,28]. Possibilities to overcome (acquired) tamoxifen resistance arose from the use of newly developed anti-estrogens[29], progestins[29] or aromatase inhibitors[30]. However, none of these agents could completely replace tamoxifen so far from first-line clinical use[28,31].

In addition to necessity to further elucidate the molecular mechanisms underlying tamoxifen resistance, it is quite important to address which therapeutic regimen could be most effective after the occurrence of tamoxifen resistance[25,26]. Therefore, in the present study we studied the expression of cyclin D1 as a proliferative marker in sensitive and resistant human breast cancer T47D cells. It is well known that specific chemotherapeutic drugs interfere with cellular pathways in one way or another. In the breast cancer cells used in our study, cyclin D1 protein expression level was different among parent and TAM-resistant cells. In addition, the growth rate of parent cells was seen to be faster than TAM-resistant cells. Therefore, observed decrease in cyclin D1 protein expression in slow growing TAM-resistant cells compared to parent cells further indicates the role of cyclins in tumor cell proliferation. It has been reported that endocrine treatment is more effective in slowly proliferating breast cancers[25]. Therefore, the anti-proliferative effect of tamoxifen on tumor growth via ER blockade may likely be due to regulation of expression of some cyclins and in particular the cyclin D1 protein.

Our results also suggest that treating breast cancer patients should be individually-based considering the tumor levels of markers such as cyclin D1. In addition, for hormone-resistant tumors, chemotherapeutic regimen should be selected based on the level of expression of tumor markers including cyclin D1 to increase the efficacy of such chemotherapy.

ACKNOWLEDGMENTS

The authors would like to thank the financial support of National Center of Excellence of Toxicology (NCET) and also kind assistance of office of vice-chancellor for research of TUMS.

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International Journal of Cancer Research 1 (1): 21-24, 2005
ISSN 1811-9727

Breast Cancer and Food: A Quasi-epidemiological Evidence of a Role for Dietary Phytoestrogens in Northwestern Nigerian Women

[1,2]Bello Shaibu Oricha, [3]Ahmed Yakubu, [4]Bello Aishatu Yahaya,
[5]Cherima Joel Yakubu and [3]Bissalah Ahmed Ekele
[1]Department of Pharmacology and Toxicology, College of Health Sciences,
Usmanu Danfodiyo University, Sokoto, Nigeria
[2]Karaye Hospital, Emir Yahaya Road, P.O.Box 1522, Sokoto, Nigeria
[3]Department of Obstetrics and Gynecology,
Usmanu Danfodiyo University Teaching Hospital, Sokoto, Nigeria
[4]Department of Obstetrics and Gynecology, Sokoto Specialist Hospital, Sokoto, Nigeria
[5]Wurno General Hospital, Wurno, Sokoto, Nigeria

Abstract: The relationship between breast cancer and dietary phytoestrogen intake in northwestern Nigerian women was explored in a case control study involving 189 cases versus 189 matched controls. Cases had significantly earlier menarche than controls and when these were adjusted for, cases significantly consumed more dietary phytoestrogens than controls. Consumption of food items like *Sorghum bicolor, Manihot esculenta* and *Typha domigensis* appear to increase the risk of breast cancer while *Oriza sativa, Mangifera indica* and *Musa sapientum* appear protective.

Key words: Breast cancer, diet, phytoestrogens, case control study

INTRODUCTION

Neoplastic diseases often have disastrous consequences on patients living in a developing economy like Nigeria. Patients typically present with late stage diseases and this may be related to illiteracy, ignorance and financial constraints, which may limit early attempts at treatment to non-orthodox practitioners. The highly limited option in treatment algorithms in developing economies also suggests that even those who present early to orthodox practices might have been missed. Given this scenario, preventive medicine remains the parsimonious option. Even this is difficult to optimize because behavioral modification is often also required and, especially in Africa, this is often restricted by highly conservative cultural values. One option is to identify and optimize beneficial factors within a cultural setting. Diet may be one such factor. Breast cancer is the commonest cancer in women[1] and is probably the commonest cancer in Nigeria. A case control study in Nigeria implicated oral contraceptive use as a risk factor for development of breast cancer[2] while current evidence suggest that daily conjugated equine estrogen (0.625 mg) combined with medroxyprogesterone acetate (2.5 mg) increase the risk of breast cancer in postmenopausal women on replacement therapy[3]. Meanwhile Soybean, rice, beans and yam are common diets in Nigeria but these have been shown to contain significant amount of dietary phytoestrogens. The impact of such diet on the risk of breast cancer in Nigerian women is uncertain. Studies in other communities and races have shown inconsistent results. Epidemiological studies comparing Asian and Western women suggest that phytoestrogens may protect against breast cancer[4] while studies in Chinese women revealed no association between phytoestrogen and breast cancer[5]. However *ex vivo* studies using concentrations equivalent to levels measured in humans consuming 40 mg of phytoestrogens supplement daily, demonstrated a stimulatory effect of phytoestrogens on Estrogen Receptor (ER)-positive breast cancer cell lines with no effect on ER-negative cells[6,7]. Difference in efficacy and potency between different phytoestrogens may be one explanation but the recent findings of lower levels of Estrogen Receptor (ER) alpha gene expression in normal breast tissue in Japanese women compared with white Australian women may be an alternative or additional explanation[8]. Whichever, it appears obvious that a global statement on the interactions between dietary phytoestrogens and breast cancer might be premature. Further studies may be important to expose

Corresponding Author: Dr. Bello Shaibu Oricha, Department of Pharmacology and Toxicology, College of Health Sciences, Usmanu Danfodiyo University, Sokoto, Nigeria
Tel: +2348035073372 Fax: +23460230450 E-mail: bellooricha@yahoo.com

ethnic differences in breast cancer rates and the association with dietary phytoestrogens. This study was carried out in northwestern Nigeria in such context.

MATERIALS AND METHODS

Three urban (Specialist Hospital Sokoto, Nigeria; Karaye Hospital Sokoto, Nigeria and Sir Yahaya Memorial Hospital Birnin Kebbi, Nigeria) and one rural (General Hospital Wurno, Nigeria) northwestern Nigerian hospital settings were selected by convenient sampling and using hospital records 189 patients who reported self as an ethnic Hausa tribe, according to Hospital records, had clinical diagnosis of advanced breast cancer with or without histopathological confirmation and who had partial or radical mastectomies were identified and either introduced to the study at the next follow up clinic or traced, if lost to follow up. Case tracing was particularly emphasized at the rural setting because of poor follow up clinic attendance but easy trace ability due to the close nit of rural communities. Cases versus 189 social level, age and parity matched neighborhood controls were interviewed using an interviewer completed 41-item questionnaire that was designed and validated in a pilot study following the method of Block *et al.*[9]. Briefly, a food list was first developed by interviewing 1329 randomly selected respondent from the community and probing them for a 24 h recall of food intake. Because only 21 food items were listed food codes were considered unnecessary. Portion size estimation was done by simple counts for unitary items (e.g. egg, oranges) and by the use of three-dimensional models for non-unitary items. In this model, abstract 3 dimensional diagrams of 5 different amounts are provided as an aid in estimating usual portion size for each food. The volume and the gram weight of the chosen model, for that particular food is pre-determined and are assigned appropriately post hoc. The questionnaire also comprised questions on demographic data including age at menarche, age at menopause, age at first full-term pregnancy and family medical history of breast cancer. To quantify the phytoestrogen content of each food item on our food list, we first translated the local Hausa name to botanical name using the list of Blench[10] and confirmed the identity by presenting samples to Crop scientists. We then conducted a literature search on Medline, Vivisimo and Agricola first by a simple query-e.g. phytoestrogen content of *Vigna sinensis* or cowpea and/or by using the common and biological names of a food item combined with all of the terms-phytoestrogens, plant estrogens, isoflavones, enterolactone, enterodiol, coumestans, lignans, daidzein, genistein, formononetin, biochanin A, coumestrol, matairesinol and secoisolariciresinol. Arithmetic Mean of published values of phytoestrogen content were determined and converted to mg/100 g food. Values reported as trace were allocated the sensitivity limit of the assay method used in that study, and food items whose values could not be obtained (2 items) were allocated values of the nearest food item determined at genus level. To estimate the average daily consumption of phytoestrogen per study subject, we multiplied the score of each food item by the serving size of the food summed across the 2 weeks food recall list per subject and then determined the arithmetic Mean. Food items may contain other unknown factor that may contribute to its overall influence on breast cancer. To test for the association of a particular food item or combinations of food items with breast cancer, we considered that direct comparison of quantity taken may give a false impression of measurement accuracy and that ranking may be more predictive. Therefore, we first determined the product of portion size and frequency of each item on the food list for each subject, and ranked this value into food-item-specific quartiles. Each subject's consumption was then allocated a quartile ranked placement as 1 (1st quartile), 2 (2nd quartile), 3 (3rd quartile) and 4 (4th quartile) per food item. The food specific modal values for cases versus controls was then determined and compared.

Statistics: Statistical analyses ware performed using Systat 10.2, Minitab 13.1 and Microsoft Excel add-in resampling statistics softwares. Dietary phytoestrogens are of low potency[11] and usually constitute a small percentage of the dietary bulk[12]; therefore we hypothesized that a difference of 30-40% in consumption between cases and control may be required for clinically observed effects. At this effect level, power analysis revealed that 176 sample per group has an 80% power at an alpha 0.05. We resampled data and checked measurements for normality by the Kolmogorov-Smirnov test. Parametrically distributed data were compared by paired t-test and ranked, scored or non-parametrically distributed data were compared by Wilcoxon matched pairs signed ranks test. Trend analyses were done using a linear regression model employing the direct values of phytoestrogen consumed and again by allocating subjects to quartile categories of phytoestrogen intake and inputting modal values of these quartile categories per food item. The association between food item and breast cancer was evaluated by binary logistic regression. We used the Mantel-Haenszel estimator and logistic regression to adjust for socio-economic class (by Tucket's classification[13]), age of menarche, age of regular menstrual cycles, age of marriage and age at first

childbirth. Level of significance was $p<0.05$ and as appropriately corrected for multiplicity when co-evaluating hypotheses.

RESULTS

Study participants include 189 cases; age 44.34±13.33 years (range 19-70 year) and 189 controls; age 41.26±17.54 year (range 19-70 year). Cases had significantly earlier age of menarche, regular menstrual cycles, age of marriage and first child birth than controls: 11±1.5 year versus 14.3±2.2 year; 11.3±1.7 year versus 16.2±3.2 year and 9.4±2.4 year versus 14.2±4.1 year, respectively. There was no significant difference between ages at menopause between cases and controls; 47.2±3.6 year versus 45.7±3.9 years, respectively.

In the study population, cases of breast cancer consume significantly more Biochanin A, Coumesterol, Diadzein, Genistein, Secoisolariciresinol than controls ($p<0.05$) (Table 1). However, the intake of Formononetin was not significantly different.

Cases significantly consumed more *Sorghum bicolor* (Sorghum), *Manihot esculenta* (Cassava), *Typha domingensis* (Bulrush), *Pennisetum glaucum* (millet), *Moringa oleifera* (Horseradish), *Hibiscus cannabinus* (Kenaf) and *Vitex doniana* (Black plum) than control ($p<0.05$) (Table 2). Consumption of *Oryza Sativa* (Rice), *Mangifera indica* (Mango) and *Musa Sapeintum* (Banana) had a hazard ratio of 0.5 (95% CI 0.3-0.7), 0.4 (95% CI 0.2-0.6) and 0.6 (95% CI 0.4-0.8) for breast cancer, respectively, while consumption of *Sorgum bicolor* (Sorgum), *Manihot esculenta* (Cassava) and *Typha domingensis* (Bulrush) had a hazard ratio of 1.3 (95%CI 1.2 -4), 1.5(95%CI 1.3-2.1) and 1.7(95%CI 1.3-2.3) for breast cancer, respectively.

Table 1: Phytoestrogen intake by cases of breast cancer versus control by northwestern Nigerian women

Phytoestrogen	Cases (Arithmetic mean and 95% CI) mg/day	Control (Arithmetic mean and 95% CI) mg/day
Biochanin A	0.13(0.10-0.15)	0.04(0.03-0.44)
Coumesterol	0.39(0.30-0.41)	0.26 (0.19-0.33)
Daidzein	2.31(2.1-2.66)	1.97 (0.99-1.11)
Formononetin	0.09(0.07-0.10)	0.10(0.07-0.12)
Genistein	2.29(1.97-2.41)	1.47(1.37-1.49)
Matairesinol	0.07(0.04-0.91)	0.03(0.02-0.04)
Secoisolariciresinol	0.21(0.17-0.26)	0.11(0.09-0.13)
Total Isoflavones	4.82(4.12-4.99)	3.58(3.14-3.76)
Total lignans	0.28(0.21-0.31)	0.14(0.12-0.16)
Total coumestans	0.39(0.30-0.41)	0.26(0.19-0.33)
Total phytoestrogens	5.42(4.99-5.62)	3.98 (3.11-4.10)

DISCUSSION

The present study revealed a demographic association between duration of exposure to natural estrogen and breast cancer . This is implied by the observation that cases had earlier menarche and regular menstruation compared to controls. The positive association of early marriage and child birth and breast cancer may have different implications in the cultural settings of Africa compared to the Western world because menarche and regular menstruations are cultural indicators of readiness for marriage in the former. Thus the observed relationship between early marriage and breast cancer may just be a surrogate for early menarche. In the African setting, early marriage is also often linked to relative dietary freedom (the women now being limited only by the husbands ability in the choice of diet). Because many African diet have high level of dietary phytoestrogen[12], such dietary freedom may translate to early onset and therefore longer duration of exposure to higher levels of dietary estrogen in cases compared to age matched controls that married latter.

Table 2: Intake of selected food by northwestern Nigerian women by cases of breast cancer versus control

Hausa name	Common name	Biological name	Cases: Modal score (and range)	Control: Modal score (and range)
Shinkafa	Rice	*Oryza sativa*	1 (1-2)	4 (1-4)
Waake	Black-eyed beans or cowpea	*Vigna sinensis*	3 (2-4)	2(1-4)
Dooya	Yam	*Dioscorea praehensilis*	1(1-3)	1(1-4)
Dawa	Sorghum	*Sorghum bicolor*	4(3-4)	1(1-2)
Jedda	Groundnut	*Arachis hypogaea*	2 (1-4)	2(1-4)
Roogo	Cassava	*Manihot esculenta*	4(2-4)	1(1-2)
Maasara	Maize	*Zea mays*	2 (1-4)	2(1-4)
Geero	Bulrush	*Typha domingensis*	4(2-4)	1(1-2)
Maiwaa	Millet	*Pennisetum glaucum*	3(2-4)	1(1-2)
Zoogaale Tomaka	Horseradish	*Moringa oleifera*	4(1-4)	2(1-2)
Ramaa	Kenaf	*Hibiscus cannabinus*	4(2-4)	2(1-2)
Kuuka	Baobab	*Adansonia digitata*	4(3-4)	1(1)
Mangwaro	Mango	*Mangifera indica*	1(1-4)	4(1-2)
Ayaba	Banana	*Musa sapientum*	2(1-4)	4(1-4)
Dunya	Black plum	*Vitex doniana*	4(1-4)	1(1-2)

Score 1-4 represent allocation to 1st, 2nd, 3rd and 4th quartile, respectively, of the consumption of an index food item. Each food item is categorized independently, e.g. category 4 of ,say, *Vitex doniana* (Black plum) is different from and has no relationship with category 4 of , say, *Musa sapientum* (Banana)

The present study also revealed that cases of breast cancer in the study region consume significantly higher amount of dietary phytoestrogen than controls, suggestion that either the type of phytoestrogen, product of intestinal metabolic activation of phytoestrogens or the estrogen receptor expression pattern of breast cancer in the study region may be different from that of in the Western world. In this regard, it may be important to recall that phytoestrogens are essentially inert until they are metabolized[14] and that studies have shown that the preferred pathways of metabolism and/or bioavailability of Isoflavones depend on the colonic microflora[15], which is expected to be richer in the African setting.

It interesting to note that *Oryza sativa* (Rice), *Mangifera indica* (Mango) and *Musa Sapeintum* (Banana), all of which contain significant amount of phytoestrogen[12], appear protective against breast cancer. This further highlights the variable effect of phytoestrogens but may suggest that when taken as part of diet, the overall effect of phytoestrogens probably depend on the overall dietary constituents. Such dietary constituent may contain antiestrogenic factors, directly influence bioavailability and/or modify intestinal microflora.

Sorgum bicolor (Sorgum), *Manihot esculenta* (Cassava) and *Typha domingensis* (Bulrush) appear to increase the risk of breast cancer in the study population. Given that these are the cheaper food item in the study area and are therefore consumed in significant amount, this association could be disturbing. However, a retrospective study is at best hypothesis generating and needs verification in prospective experimental studies. This may be difficult to design in human but *in vitro* carcinogenic studies and *in vivo* studies in animal models may better guide the context which these diet are placed.

This study provides evidence that further support a relationship between phytoestrogen and breast cancer and suggest a complex relationship that may be dictated by factors other than the phytoestrogen. Such factors probably include the contest in which the phytoestrogen is ingested. This may not be surprising considering that the biological effect of native estrogen is also complex[16].

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International Journal of Cancer Research, 1 (1): 25-28, 2005
ISSN 1811-9727

Communications of Neu/c-erB-2 Receptor and Inhibits Growth of Human Malignant Glioma Cell Lines Following Tamoxifen Treatment

[1]Mohd Nazil Salleh, [1]Chew Yee Choy, [1]Patimah Ismail, [2]Norashikin Shamsudin and [3]Paul Carmichael
[1]Molecular Biology and Cell Unit, Department of Biomedical Sciences,
[2]Internal Medicine Unit, Department of Medicine, Faculty of Medicine and Health Sciences,
Universiti Putra Malaysia, 43400 UPM Serdang Selangor, Malaysia
[3]Division of Biological Sciences, Imperial College Medicine Science and Technology, South Kensington,
London SW7 2AZ United Kingdom

Abstract: In the present study, effects of tamoxifen, an antiestrogen, on the inhibition of protein tyrosine phosphorylation in neu/c-erbB-2 receptor, DNA synthesis and proliferation were evaluated using the malignant glioma cell lines U25 IMG and T98G which overexpressing neu/c-erbB-2. Pre-treatment of two cell lines with tamoxifen resulted in a dose dependent inhibition of tyrosine phosphorylation as well as DNA synthesis and cell growth in two cell lines correlatively. The results support the hypothesis that activated protein tyrosine kinase receptors are involved in the proliferation of glioma cells. Tamoxifen may be useful in the treatment of malignant glioma.

Key words: Tamoxifen, neu/c-erB-2, protein tyrosine phosphorylation, DNA, glioma

INTRODUCTION

The human neu/c-erbB-2 proto-oncogene encodes a 185 kilodalton (kD) transmembrane glycoprotein, neu protein, with intrinsic tyrosine kinase activity that resembles the Epidermal Growth Factor Receptor (EGFR)[1,2]. The neu/c-erbB-2 gene and protein were originally identified in the brain and are thought to play a critical role in neurogenesis. The overexpression of the gene for the neu protein appears to be correlated with aggressiveness of various tumors[3,4] Protein Tyrosine Kinase (PTK) receptors that have been implicated in a wide variety of physiological functions and tyrosine kinase are involved in the signal transduction cascades from the growth factor receptor towards the deoxyribonucleic acid (DNA) replication system in the nucleus. An imbalance in the phosphorylation state of signal-transducing proteins is thought to contribute to deregulated cell proliferation. In several types of tumors, increased protein PTK activity was found. In some tumor, PTK activity correlated with histology, lymph node metastasis, or prognosis. Tamoxifen, (Z)-2[p-(1,2-diphenyl-1-butenyl) phenoxy]-N, N-dimethylethylamine citrate (1:1), a synthetic, nonsteroidal antiestrogenic compound, which has been used extensively in the treatment of estrogen receptor (ER)-positive breast cancer[5-7]. Tamoxifen was reported to have inhibited the growth of some ER-negative cell lines of malignant glioma[8,9] It has been reported that tamoxifen inhibits protein kinase C (PKC) by interfering with its activity of the catalytic subunit[9-11]. Tamoxifen can also penetrate the blood-brain barrier[11], which suggests a possible role for it in the treatment of malignant glioma[8]. We report here the effects of tamoxifen on the tyrosine phosphorylation of neu protein and its influence on the DNA synthesis and proliferation in two human glioma cell lines.

MATERIALS AND METHODS

Cell culture: The human glioblastoma cell line T98G and U-25 IMG were obtained from the Molecular Toxicology Unit, Imperial College, London. Cells were cultured as monolayers in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated FCS, 15 mmol L^{-1} L-glutamine, 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in a humidified 5% CO_2 atmosphere at 37°C. For maintenance of the cells under serum-free conditions, the medium was replaced by the chemically defined MCDB 10^5 medium (Sigma).

DNA synthesis: Cells were seeded into 96~well microtiter plates (10^4 cells/well) and cultured in DMEM, 10% FCS for

Corresponding Author: Dr. Mohd Nazil Salleh, Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
Tel: (+603)-8946 8475 Fax: (+603) 8943 6178 E-mail: nazil@medic.upm.edu.my

12 h. Subsequently, cells were washed twice in serum-free MCDB 10^5 medium and grown for 24 h under serum-free conditions. The cells were finally incubated for another 48 h with various concentrations of tamoxifen (Sigma) (1 ng mL^{-1} to 10 µg mL^{-1}). Control cells were maintained in MCDB 10^5 without tamoxifen. [3H] thymidine (1.0 µCi/mL) was added for the last 12 h. Cells were washed with Phosphate-buffered Saline (PBS), detached by a 15 min incubation with 0.050/0 trypsin, 0.02% ethylene diaminetetraacetic acid in PBS at 37°C and harvested onto filters. The filters were extensively washed and radioactivity was quantified in a liquid scintillation counter.

Cell proliferation: Cells were seeded into 96-well microtiter plates (5×10^3 cells/well) in serum supplemented medium. After overnight incubation, the mediums were replaced with MCDB 10^5 and allowed to grow for 12 h. The mediums were then changed into fresh MCDB 10^5 (as control) or into mediums containing tamoxifen at concentrations of 50 and 200 ng mL^{-1}. Cells were detached with 0.25% trypsin in Hanks balanced salt solution and counted in a hemocytometer after 48, 72 and 96 h. Cytostatic test was performed with 0.2% trypan blue solution and the unstained viable cells were microscopically distinguished from the blue stained damaged cells.

Tyrosine phosphorylation of neu receptor: Cells were seeded in 10 cm tissue culture dishes and grown in DMEM containing 10% FCS. The cells were washed and incubated for 24 h with serum-free MCDB 105 medium. Various concentrations of tamoxifen (1 ng mL^{-1} to 10 µg mL^{-1}) were added and the dishes were incubated at 37°C for 10 min. The plates were washed with ice-cold PBS, including 100 µmol L^{-1} sodium orthovanadate. Cells were scraped into 0.5 mL of lysis buffer and lysates were centrifuged at 50,000 x g for 30 min at 4°C. Solubilized fractions of $1x\ 10^6$ cells were incubated for 2 h with rabbit anti-neu antibody (c-neu, Oncogene Science, Inc). The immunoprecipitates were collected with Protein A-Sepharose beads and washed with HNTG buffer (20 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.5, 150 mmol L^{-1} NaCl, 10% glycerol, 0.1% Triton X-100) which was supplemented with 1 mmol L^{-1} sodium orthovanadate. The immunoprecipitated protein was separated by SDS-polyacrylamide gel electrophoresis (PAGE) in 7.5% gels and blotted with anti-phosphotyrosine antibody PY20 (1 µg mL^{-1} 2 h, Sigma). The tyrosine-phosphorylated proteins were detected using horseradish peroxidase (HRP) ABC kit (Sigma) and visualized with the ECL chemiluminescence method (Amersham Corp).

Statistical analysis: All experiments were performed three times. Statistical significance of thymidine uptake and cell growth was assessed by unpaired Student's t-test. All samples were done in triplicate.

RESULTS

Effect on DNA synthesis:[3H] thymidine uptake was reduced markedly in both U25 1-MG and T98G cell lines after exposure to 1 to 1000 ng mL^{-1} tamoxifen for 48 h. The inhibition effect of tamoxifen displayed a dose-dependent pattern. At concentrations of tamoxifen greater than 200 ng mL^{-1} [3H] thymidine incorporation was less than 5% of control levels in two cell lines.

Effect on cell proliferation: After two cell lines were treated with 50 and 200 ng mL^{-1} tamoxifen, cell numbers were determined after 48, 72 and 96 h (Fig. 2). Cell growth was also inhibited by tamoxifen in a dose-dependent pattern. After 48h, 50 ng mL^{-1} tamoxifen reduced cell numbers to less than 50% (42±3.0% of U251-MG and 39±2.5% of T98G) ($p<0.001$) and 200 ng mL^{-1} tamoxifen further reduced cell number to less than 25% (23±2% of U251-MG and 15% ±3.2 of T98G) ($p<0.001$) of the controls. At 200 ng mL^{-1} tamoxifen produced near complete inhibition of proliferation in each of the cell lines after 96 h. The effect achieved by this concentration was cytostatic rather than cytotoxic because more than 95% of cells excluded trypan blue[9].

Effect on tyrosine phosphorylation of the neu receptor: The interaction of tamoxifen with neu receptor in the cell lines was confirmed by assaying the receptor tyrosine phosphorylation after having treated it with 10, 50 and 200 ng mL^{-1} tamoxifen. Immunoprecipitation of neu receptor protein (185 kDa protein) from tamoxifen treated

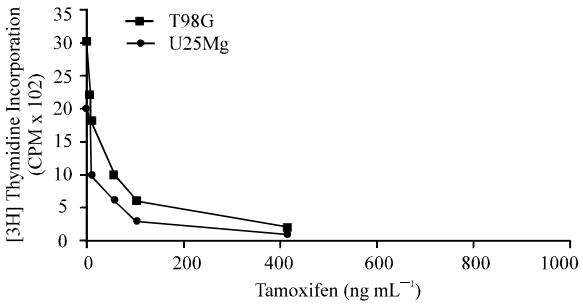

Fig. 1: [3H] thymidine uptake in two human glioma cell lines (U2S l-MG. T98G) after exposure to tamoxifen (1 ng mL^{-1} to 10 µg mL^{-1}) for 12 h. Each point represents the average of triplicate determination

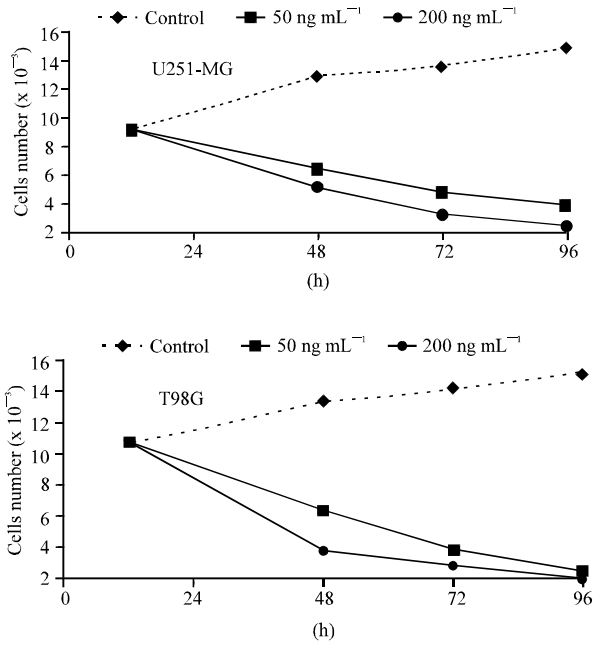

Fig. 2: Graphs showing cell growth curves in two glioma cell lines (U25 l-MG, T-98G) treated with tamoxifen in 50 ng mLG^{1} and 200 ng mLG^{1} for up to 96 h

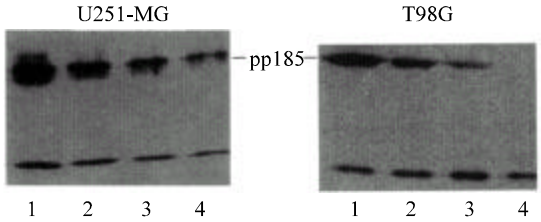

Fig. 3: Tyrosine phosphorylation of the neu receptor. Cell lines U251-MG and T9gG were incubated without (lane 1) or with 10 ng mLG^{1} (lane 2), 50 ng mLG^{1} (lane 3), 200 ng mLG^{1} (lane 4) of tamoxifen for 10 min at 37°C. The neu receptor was immunoprecipitated with anti-neu receptor antibody and immunoblot analysis performed with anti-phosphotyrosine antibody. Positions of coelectrophoresed protein markers in kD are indicated

Cells was followed by immunoblotting with anti-phosphotyrosine antibody. Tyrosine phosphorylation of neu receptor protein in two cell lines was inhibited by various concentrations of tamoxifen in a dose-dependent pattern (Fig. 3). The reduction of phosphorylated tyrosine protein, at 10, 50 and 200 ng mLG^{1}, was 3, 5 and 10 fold in U251MG cell and it was 3, 10 and 0-fold in T98G cell. In T98G cell line, the tyrosine phosphorylation was extremely inhibited and no phosphorylation was detected at 200 ng mLG^{1} tamoxifen.

DISCUSSION

The PTK receptors are known to be involved in regulation of cell growth, cell differentiation, chemotaxis and actin reorganization[12]. Phosphorylation of p185-neu on tyrosine could result from autophosphorylation or from phosphorylation by a distinct tyrosine kinase. Autophosphorylation of p185-neu could be activated by binding of a ligand to the external domain, or by clustering and consequent intermolecular interactions of pl85-neu monomers concentrated at high densities at the plasma membrane[13-15]. In the present study the tyrosine phosphorylation of neu receptor as well as DNA synthesis and cell proliferation in the malignant glioma cell lines U25 l-MG and T98G was markedly inhibited by tamoxifen. The magnitude of this receptor phosphorylation correlated with the subsequent onset of DNA synthesis. These results suggest that tamoxifen interacts with neu receptor and inhibits tyrosine phosphorylation of neu receptor. It also shows that protein tyrosine phosphorylation of neu receptor is probably a critical factor in the proliferation of glioma. We infer the inhibition of neu receptor tyrosine phosphorylation resulted in blocking the signal transduction cascades from the receptor toward the DNA replication, proliferation and aggressive growth associated with malignancy[14]. The anti-estrogen tamoxifen has long been used in the treatment of postmenopausal women with ER-positive breast carcinoma[5-7] and is a potent inhibitor of the proliferation of ER-positive tumors *in vitro*. Studies showed that the effect of this agent and its metabolites in such tumors was largely mediated by competitive inhibition of estrogen binding to its receptor. Tamoxifen is also known to be a PKC inhibitor that effected cell proliferation by pathways independent of the ER[2,5,11,16].

The inhibition achieved by tamoxifen in these cell lines was demonstrated at concentrations that were achieved therapeutically in sera of patients with breast cancer (100 to 2000 ng mLG^{1})[12,17,18]. Because tamoxifen can also penetrate the blood-brain barrier (BBB)[18] , it has been successfully used in the treatment of cerebral metastases from breast cancer[19,20] and to inhibit ER-mediated effects in various cell populations with the central nervous system that lie behind the BBB[20,21] tamoxifen can be a potential agent in the treatment of malignant glioma. In conclusion, tamoxifen has proven to be a potent inhibitor of human glioma proliferation *in vitro*, possibly by inhibiting protein tyrosine (auto) phosphorylation in neu receptor. It has the potential to be

a useful agent in the treatment of glioma. Further research will carry on the mechanism of inhibition on the tyrosine phosphorylation in neu receptors.

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International Journal of Cancer Research 1 (1): 29-35, 2005
ISSN 1811-9727

Measurements of the Optical Properties of Breast Tissues *in vitro* Using Mueller Matrix Polarimetry

Shamaraz Firdous, Masroor Ikram and M. Faisal
Department of Physics and Applied Mathematics,
Pakistan Institute of Engineering and Applied Sciences,
P.O. Nilore 45650, Islamabad, Pakistan

Abstract: Laser transmittion, absorption and scattering technique for photon migration in human breast tissues phantom with Mueller matrix polarimetry have been investigated in this study. Polarized laser transmittion, including depolarization of wave applied to biological tissues provide a comprehensive frame work and simple way for diagnostic and treatment of skin lesion. He-Ne Laser (8=632.5 nm) imaging system is described for non invasive and non radioactive clinical procedure. The system generates 16 full out put Mueller matrices for characterization of tissues. This matrix along with optical images can characterize the normal and malignant tissues for diagnostic as will as treatment procedures of breast cancer. Although the *in vitro* measurements are smaller then *in vivo*, but this research work provides a base for designing quick model of polarized laser tissues culturing and imaging.

Key words: Optical imaging, birefringence, depolarization, laser scattering, breast phantom, Mueller calculus

INTRODUCTION

Human tissue is relatively transparent to visible light in the bandwidth between 600 and 1000 nm, so that light can penetrate into tissue to a depth of up to several centimeters depending on the type of tissue. This property can be used to obtain physiological information, for example to detect tumors or to measure hemodynamic changes in tissue[1]. The determination of the optical absorption and scattering properties of tissue is of great interest for the noninvasive diagnosis by light. The particular interest in this area is the transillumination of the female breast for a preventive checkup[1,2], light screening could be an alternative to conventional x-ray mammography or could yield additional diagnostic information. The transilluminating living tissues like the female breast, the measured overall spectral characteristics are determined by recording different constituents with different wavelength dependencies of the corresponding optical parameters. Particularly the major components involved are water, fat and blood, other chromospheres for instance glucose, cytochrome and melanin are less important because their signals are very weak[3,4]. Light as a medical diagnostic tool for *in vivo* and *in vitro* tissue characterization has been used widely due to its noninvasiveness and non radioactive[5,6]. Because of the important information that light reveals, optical imaging has found many applications in biological tissue measurements[7,8]. Optical imaging has been used to probe surface structures, such as the functional activation of the exposed brain regions, skin cancers and those revealed by endoscopic procedures, but it has also been used to investigate noninvasively the internal function of large organs such as the breast[9-14]. Polarimetry is powerful tools that have been applied in various disciplines. The research in advanced polarimetry for the development of biomedical diagnostic and treatment using polarization effects of the scattered light and characterization of the samples was started[15]. The scientist have shown that polarization-based imaging measurements can provide an enhancement in superficial structures to allow for subsurface imaging[16,17]. These applications of polarimetry for biomedical imaging involve the use of Mueller-Stokes calculus to mathematically depict how a biological sample affects the polarization vector of an incident light beam, determined by either backscattered or transmitted light intensities from the sample[18,19].

Optical imaging may have a major role in breast cancer research and detection, despite its low resolution, by assessing functional and molecular cancer characteristics. The unique features of the optical method,

Corresponding Author: Shamaraz Firdous, Department of Physics and Applied Mathematics,
Pakistan Institute of Engineering and Applied Sciences, P.O. Nilore 45650, Islamabad, Pakistan
E-mail: sfirdous@pieas.edu.pk

Table 1: Calculation of the 16-image Mueller matrix. The notation is as follows: the first term represents the input polarization state, while the second term represents the output polarization state. The states are defined as: H for horizontal, V for vertical, P for+45°, M for-45°, R for right circular and L for left circular

M_{11}=HH+HV+VH+VV	M_{12}=HH+HV-VH-VV	M_{13}=PH+PV-MH-MV	M_{14}=RH+RV-LH-LV
M_{21}=HH-HV+VH-VV	M_{22}=HH-HV-VH+VV	M_{23}=PH-PV-MH+MV	M_{24}= RH-RV-LH+LV
M_{31}=HP-+HM+VP-VM	M_{32}=HP-HM-VP+VM	M_{33}=PP-PM-MP+MM	M_{34}= RP-RM-LP+LM
M_{41}=HR-HL+VR-VL	M_{42}=HR-HL-VR+VL	M_{43}=PR-PL-MR+ML	M_{44}= RR-RL-LR+LL

along with its high sensitivity for detecting photons and use of nonionizing radiation, renders optical imaging a technology that could complement existing breast imaging techniques for cancer detection and characterization. The compatibility of the technology with most other radiological imaging techniques allows the creation of combined modalities for simultaneous breast examinations that yield a superior feature set. Furthermore, optical methods are economic and can acquire data continuously, hence, they may be used for real-time monitoring[20].

Background

Optical parameters of breast tissue: The attenuation of a light beam of intensity in an absorbing solution can be described by Lambert-Beer's law. The absorption coefficient u_a is directly proportional to the concentration of the absorbing particles. In contrast, in a highly scattering medium such as biological tissue, the photons travel several times the geometric thickness of the specimen. Light attenuation in a scattering medium can be described by the scattering coefficient u_s. The anisotropy factor g describes the anisotropic distribution of the angles of light scatter, i.e. the mean cosine of the scatter angle and typical values for biological tissue range between 0.9 and 1. For more detail we can study the literature contain references on the optical parameters of breast tissue *in vivo* and *in vitro*[21-24].

Mueller matrix: To specify the polarization configuration of a radiation beam, the Stokes parameters S_0,S_1,S_2,S_3 are required. The Stokes parameters are defined as follows[25]:

$$\begin{aligned} S_0 &= \langle E_{0x}{}^2 + E_{0y}{}^2 \rangle \\ S_1 &= \langle E_{0x}{}^2 - E_{0y}{}^2 \rangle \\ S_2 &= \langle 2E_{0x}E_{0y}\ \cos d \rangle \\ S_3 &= \langle 2E_{0x}E_{0y}\ \sin d \rangle \end{aligned} \quad (1)$$

where, S_0 is the total detected light intensity which corresponds to the addition of any of the orthogonal component intensities, while S_1 is the portion of the intensity that corresponds to the difference between the square of the linear horizontal and vertical polarization states, S_2 is the portion of the intensity that corresponds to the square of the difference between the linear +45° and -45° polarization states and S_3 is the portion of the intensity that corresponds to the square of the difference between the right circular and left circular polarization states. In the present study the Mueller matrix associated with the single order transmittion by turbid phantom is studied. For simplicity, we assume that nonspherical particles are randomly oriented in space. For randomly oriented particles, having a plane of symmetry, the scattered Stokes parameters are related to their incident counterparts by the phase matrix in the following form

$$[M]=\begin{bmatrix} M_{11} & M_{12} & M_{13} & M_{14} \\ M_{21} & M_{22} & M_{23} & M_{24} \\ M_{31} & M_{32} & M_{33} & M_{34} \\ M_{41} & M_{42} & M_{43} & M_{44} \end{bmatrix} \quad (2)$$

As shown in Fig. 1, we consider a collimated radiation beam illuminating a thin layer composed of particles along the z-axis direction. For simplicity, the particles concentration is assumed to be low i.e., the optical thickness for the layer is small so that the scattered energy is essentially contributed by the first-order scattering events. One method of observing the scattered Stokes parameters is use of optical detectors or CCD camera. If such an array was placed on the x, y plane, the backscattered Stokes parameters could be related to the incident Stokes parameters as follows:

$$[S_{out}] = [M]\,[S_{in}] \quad (3)$$

where, both the incident and the scattered Stokes parameters need to be specified with a fixed laboratory reference plane. Polarized light can be completely described using the 4x1 Stokes vector, as demonstrated in Eq. 1[26]. The Mueller matrix is a mathematical representation of the optical polarization properties of a given sample in which the Stokes vector of a probing light source can be combined with the Mueller matrix of the sample to determine the polarization state of the detected output beam. knowing the input light polarization state, S_{in} and the 16 Mueller matrix element of the sample, one can determined S_{out} for a given sample (Table 1). Since biomedical imaging applications include measurements in transmission and diffuse reflectance modes, we modeled and tested our system for each of these modalities and

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International Journal of Cancer Research 1 (1): 36-40, 2005
ISSN 1811-9727

The Inhibitory Effects of Nicotine on Azaserine Initiated Rat Pancreatic Carcinogenesis

Haydar Oztas and Deniz Yildiz
Department of Biology, Faculty of Science and Art, Mustafa Kemal Üniverstiy, Antakya-Hatay, Turkey

Abstract: The objective of the present study was to investigate the tumor promoting effects of nicotine on exocrine pancreas in azaserine-rat model. Two weeks old male Leeds strain rats were given a weekly i.p. injection of azaserine (30 mg kgG^{1} body weight) for five weeks. The other group was untreated with azaserine and kept as control groups. At the end of five weeks of treatment, rats were divided into four groups each group including 15 rats and exposed to nicotine for six months. At the end of the experiments, the animals were killed, the bodies were weighted and the pancreases were excised and weighted. The pancreases were kept in 10% formaldehit for histological processes. The results of this study showed that exposure of azaserine for five weeks initiated the pancreatic cancer in rats. The standard diet plus nicotine mixture had an inhibitory effect on acinar cells of rat exocrine pancreas. The observed reduction in stereological parameters such as volume of Atypical Acinar Cell Foci (AACF) and mean focal diameter of foci (AACF) was significant ($p<0.05$) compared to azaserine initiated control rats. The results of the present study do not provide any evidence for that nicotine promotes exocrine pancreatic carcinogenesis in rat pancreas. Nicotine rather seems to be associated with the reduction of AACF that is produced by azaserine injection.

Key words: Nicotine, rat, pancreas, carcinogenesis

INTRODUCTION

Several epidemiological studies have suggest that cigarette smoking is associated with a high risk of pancreatic cancer occurrence[1]. It is well known that pancreatic cancer continues to be a leading cause of death in men and women worldwide[2]. Peto *et al.*[3,4] have estimated that in developing countries, there are about two millions of death attributed to cigarette smoking. Previously, nicotine has not been shown to have carcinogenic effects, but it is suspected that nicotine may promote tumor growth in the lung[5]. Shizonuka *et al.* [6] were the first to compare the efficiency of tobacco smoking on pancreas carcinogenesis. In a study, researchers detected a close association between tobacco smoking and AACF development in exocrine pancreas. It is well known that this kind of AACF may lead to exocrine pancreas in rats[7]. Hecht *et al.*[8] claimed that smokers are repeatedly exposed to carcinogenic and genotoxic substances such as polynuclear aromatic hydrocarbons (PHAs), benz [a] pyrene and the nicotine-derived tobacco specific nitrosamine, 4-methylnitrosamine-1-(3-pyridyl-1-butanone) (NNK) which are powerful pulmonary carcinogens in rodent at doses similar to those encountered in a lifetime of smoking. Although the carcinogenic effects and metabolic pathways for PAH and NNK are well known, the carcinogenic effects and metabolic pathways for nicotine are not well described. In respect of pancreatic carcinogenesis, a tobacco smoke component, nicotine appears to be particularly important because of its relatively high level in cigarette smoke. Previously, it has been shown that a typical cigarette contains 6-11 mg of nicotine, of which the smokers can absorb 1-3 mg that is sufficient to establish and sustain nicotine dependence[9,10]. Performed researches have shown that nicotine acts on the neuronal nicotinic acetylcholine receptor (nAChR) which is a ligand-gated ion channels mediating the cholinergic neurotransmission[11]. Maneckjee and Minna[12] observed that some lung cancer types express nAchRs. Low concentrations of nicotine blocked the induction of apoptosis in these cells. So far researchers have evaluated the biochemical effects of nicotine and NNK on cultures of normal human bronchial epithelial cells[6], but there is no study about the carcinogenic properties of nicotine on pancreas. For this reason, the initial objective of this study was to determine whether nicotine could promote exocrine pancreatic carcinogenesis in a well-known model, azaserine-rat model. One of pharmacologically important component of tobacco smoke component, nicotine, could provide further insight into the understanding carcinogenic effects of tobacco smoke.

Corresponding Author: Dr. Deniz Yildiz, Department of Biology, Faculty of Science and Art, Mustafa Kemal Üniverstiy, Antakya-Hatay, Turkey Fax: 03262455867 E-mail: dyildiz@mku.edu.tr

MATERIALS AND METHODS

Chemicals: Azaserine with a purity of 98% (evaluated by thin layer chromatography) was purchased from Sigma Chemical Co. (USA) Formalin and acetone was obtained from BDH Chemicals Ltd. (UK).

Animals: Male inbred Leeds strain rats were obtained from our breeding colony. The rats were maintained under standard conditions (room temperature 23°C; lighting 7 am - 7 pm) on sawdust bedding. Rats fed with standard diet (Paterson and Christopher Hill Group, Protein Diet, PRD) plus 500 mg kg^{-1} (equivalent to 10 mg/rat/day) nicotine (one of control and experimental group) or only standard diet (one untreated control, one azaserine initiated control group). During the feeding process nicotine group's standard diet was mixed with nicotine with a V-shaped blender for one hour to obtain a homogeneous preparation. For this purpose, nicotine was first dissolved in a minimal quantity of acetone prior to mixing with standard diet to a final concentration of 500 mg kg^{-1} weight and then added to the standard diet. The prepared diets were stored separately in dark in a sealed container. Animals had access to food and water *ad libitium* and the powder feeders were replenished with fresh diet twice a week.

Starting at two weeks of age, totally 30 male rats received a weekly i.p. injection of 30 mg kg^{-1} of azaserine for 5 weeks. The same dose of 0.9% NaCl solution injected by the same method to other 30 control rats. At the end of seven weeks, animals were divided into four different groups as the following; The first group (UnCt) received no azaserine treatment. Rats in this group were fed with only standard diet. The second group (AzCt) included the azaserine injected rats and was kept as control rats. The third group (NiCt) received no azaserine but was treated with nicotine. Rats in this group were fed with a mixture of 500 mg kg^{-1} nicotine diet. The fourth group (AzNi) included the azaserine injected and nicotine treated rats. Rats in this group were fed with a diet containing 500 mg kg^{-1} of nicotine. Animals were healthy at the end of experimental period. Animals were then killed at the end of six months.

Stereological analysis of pancreases: The pancreases were excised by autopsy and all of the adherent fat, the mesentery and the lymph nodes were carefully trimmed off. The wet weight of each pancreases was recorded before fixation in 10% buffered neutral formalin for approximately 8-18 h. Before immersion in the fixative solution, each pancreas was spread out on a piece of porous paper to ensure maximal transactional area for subsequent sectioning. Thus, a single section of maximal area was obtained for stereologicy for each pancreas. Sections were then cut into 5 µm on a microtome and stained with haematoxylin-eosine. Acidophilic foci in sections were identified and classified according to established criteria[13]. The total area of exocrine pancreatic tissue was measured directly in a single histological section from each pancreas by means of a VIDS III video image analyzer (Analytical Measuring Systems, Cambridge). The same instrument was used to count acidophilic and basophilic AACF and measured their transactional areas. The observed data was processed numerically by a computer software package (Volugen), which uses an algorithm based upon the mathematical formula of Campbell *et al.*[14] as modified by Pugh *et al.*[15]. In this model, the foci with areas below reliably detectable values are subtracted from total number of intersections counted.

Statistical analysis: Numbers of pancreatic AACF, mean values and standard error of means were determined for all data. Non parametric statistical analysis were performed using the one-way analysis of variance, the Mann-Whitney U-Test.

RESULTS

Body and pancreatic weights: The mean weight of the rats in the UnCt, AzCt, NiCt and AzNi groups did not show a significant difference (Table 1). The mean pancreatic weights of all of the rat groups also did not show any statistical differences. During a gross examination of pancreas, no pathologic changes related to nicotine toxicity was observed.

Quantitative analysis of foci: Quantitative analysis of atypical basophilic and acidophilic cell foci, AACF, developed during experimental processes in rat pancreas is shown in Table 2 and 3. As expected, UnCt had a very low rate of atypical acinar cell foci (acidophilic and basophilic) that arose spontaneously on rat pancreas. NiCt pancreases had slightly higher atypical acinar cell foci compared to UnCt. However, the differences between these groups did not reach to a statistically significant level ($p<0.05$). AzNi represented a greater number of basophilic atypical acinar cell foci per mm^2 (0.528 Vs 0.463) compared to AzCt (Table 2). Some minor differences were also found between these groups with respect to the number of foci per pancreas (692.11 Vs 550.00), mean focal volume of foci (0.072 Vs 0.11) and volume of foci as a % of pancreas (0.383 Vs 0.456) but differences was not statistically significant (Table 2). Comparison of Atypical

Table 1: Effects of nicotine feeding, following initiation with azaserine, body and pancreatic weights (g). (No statistical differences between groups)

Groups (n=15)	UnCt	AzCt	NiCt	AzNi
Body weight (g)	326±19.8	342.70±17.6	302.50±33.6	266.30±16.7
Pancreatic weight (g)	1.25±0.31	1.35±0.166	1.26±0.179	1.31±0.152

Table 2: Effects of nicotine feeding, following initiation with azaserine, on induction of Atypical Basophilic Acinar Cell Foci (AACF)

Groups (n=15)	UnCt	AzCt	NiCt	AzNi
No. of AACF per mm^2	0.0004±0012	0.022±0.013	0.0012±0.0025	0.0985±0.0272
No. of AACF per mm^3	0.0071±0.023	0.463±0.025	0.0024±0.0125	0.528±0.163
No. of AACF per pancreas	4.1200±5.18	550.000±248.51	6.2300±12.46	692.110±232
Volume of AACF as % of pancreas	0.0020±0.005	0.456±0.243	0.0040±0.002	0.383±0.188
Mean focal diameter (mm)	0.7100±0.416	0.260±0.02	0.2100±0.06	0.240±0.085
Mean focal volume (mm^3)	0.0032±0026	0.110±0.008	0.0020±0.001	0.072±0.007

Table 3: Effects of nicotine feeding, following initiation with azaserine, on induction of Atypical Acidophilic Acinar Cell Foci (AACF)

Groups (n=15)	UnCt	AzCt	NiCt	AzNi
No. of AACF per mm^2	0.0004±0.0012	0.0369±0.013	0.0015±0.0018	0.0233±0.0156
No. of AACF per mm^3	0.0015±0.0037	0.0628±0.0259	0.0022±0.0081	0.0638±0.044
No. of AACF per pancreas	0.5500±1.74	90.1800±43.38	3.5100±10.53	69.2400±231.55
Volume of AACF as % of pancreas	0.0010±0.001	0.9730±0.539	0.0050±0.010	0.0320±0.216 * Vs AzCt
Mean focal diameter (mm)	0.3200±0.09	0.6800±0.16	0.2100±0.06	0.4700±0.192 *Vs AzCt
Mean focal volume (mm^3)	0.0050±0.001	0.1760±0.125	0.0030±0.004	0.0660±0.056

Acinar Cell Foci (AACF) parameters of UnCt with NiCt indicates that NiCt rats are higher than UnCt rats (3.51 Vs 0.55), but differences did not reach to significant level ($p<0.05$). The other parameters observed for both groups (NiCt Vs. UnCt) did not show any significant differences ($p<0.05$). When AzNi is compared with AzCt, AzNi represented a reduced quantitative parameter (Table 3). The differences on the volume of atypical acinar cell foci (AACF) (0.032 Vs 0.973) and mean focal diameter of foci (AACF) (0.47 Vs 0.68) were statistically significant ($p<0.05$). Also the volume of AACF as % of pancreases were reduced by 7.76%, but the differences did not reach to a significance level.

DISCUSSION

Tobacco smoke contains at least 43 compounds known to induce tumors in laboratory animals and tobacco smoking is a well-established risk factor for lung cancer[16]. It has been shown that nicotine derived compounds such as 4-methyl (nitrosamine)-1-(3-pyridyl)-1-butanone (NNK) may cause neoplastic development especially in lungs of laboratory animals[17]. But so far, carcinogenic effects of nicotine and related compounds in exocrine pancreatic carcinogenesis are not shown. Previous investigations of epidemiology suggest that tobacco smoking is associated with increased pancreatic carcinogenesis[2]. The first objective of this study was to determine the possible promoting effects of nicotine by evaluating the AACF formation that occurs following long-term exposure of smokers to the carcinogens. But the results of this investigation have shown that a six months exposure of rats to a nicotine mixed diet did not promote AACF development in exocrine pancreas. In contrast, our results claim that nicotine may reduce the development of preneoplastic AACF development which potentially leads to pancreatic carcinogenesis. Quantitative stereological parameters of basophilic AACF in AzNi group was increased when compared with other groups, but differences were not significant. Basophilic AACF seems to have only a low potential for growth and progression to neoplasm, whereas a higher fraction of lesions that classified as acidophilic seem to have the potential for such a progression[13]. Rao *et al.*[13] have reported that the size of basophilic AACF tends to remain constant, that they have a consistently low or absent proliferative capacity. Acidophilic AACF have been previously observed in low numbers by several investigators in normal aging rats not exposed to any chemical carcinogen[18]. It has been reported that incidence of these acidophilic AACF increase with age[19]. The initiating effects of azaserine is well known on rat exocrine pancreas. In the early stage it mostly causes the development of precursor AACF and tumors. In our study, we observed a decreased AACF development in AzNi rats. The decreases in focal parameters in AzNi rat pancreases indicates that at least in the light of this findings nicotine cannot be a promoter of atypical acidophilic cell foci. The observed decrease in quantitative parameters of acidophilic AACF showed that in exocrine pancreas, nicotine is somehow effective in reduction of atypical acinar cell foci development in the early stage of the experiment. To interpret this reducing effect of nicotine on AzNi on AACF is difficult. Brognard *et al.*[20] found that the expression of nicotinic acetylcholine receptor subunite types, timing and duration of the Akt response and effects of Akt phosphorylation of downstream substrates varied

between normal human bronchial epithelial cell and small airway epithelial cells following stimulation with nicotine or NNK. Nicotine also caused a loss of contact inhibition at high cell densities in culture and when lung epithelial cells were challenged with a variety of apoptotic stimuli, nicotine and NNK inhibited the induction of apoptosis[20]. It was speculated that based on the early studies phosphorylation of Akt on the physiologically relevant serine and threonine residues was active in lung cancer cells derived from smokers and hypothesized that compounds such as nicotine might affect the Akt pathway in normal lung cells. It has been suggested that activation of this signal transduction pathway may change the behavior of normal lung epithelial cells, making them more similar to cancer cells[21]. But there is not any finding about activation of Akt pathway on normal pancreatic cells. It is well known that serine/threonine kinase Akt pathways regulated various cellular activities, such as cell growth and apoptosis. Akt is a multifunctional serine-threonine protein kinase which is in a low-activity conformation in quiescent cells cytosol. In this study, it is possible to speculate that possible lack of metabolic activation of nicotine by pancreatic acinar cells for Akt could be partially responsible for reduction of atypical acinar cell foci parameters. But further research is needed about the biochemical activity of Akt in exocrine pancreatic carcinogenesis. It could be interesting to find out how nicotine or carcinogenic derivatives of nicotine acting through the nicotinic acetylcholine receptors in acinar cells cause a loss of contact inhibition and development of resistance to apoptosis. Previous studies have demonstrated that the initiating/promoting effects of polycyclic aromatic hydrocarbons, heterocyclic amines and N-nitrosamines in lung carcinogenesis, but carcinogenic effects of this substances on pancreatic carcinogenesis remain to be elucidated. Henningfield *et al.*[22] demonstrated that a typical cigarettes contain 6-11 mg of nicotine of which the smokers can absorb 1-3 mg. The researchers found that nicotine and NNK, at concentrations equivalent to those reached in bloodstream of smokers, activated the Akt pathway, but mechanism by which the two compounds acted was different. This study provides that the development of pancreatic carcinogenesis depending on tobacco smoke seems to be more complex than previously thought[5]. A decrease in the focal parameters of azaserine initiated–nicotine fed rats may indicate that at least nicotine cannot be a promoter of atypical acinar cells developed after azaserine treatment. It is not always possible to ascribe these variations to any specific experimental conditions, although the total dosage of azaserine can be expected to increase the number of foci. Additional research would be required using various doses of nicotine to elucidate whether it is a promoter or non-promoter in exocrine pancreas.

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International Journal of Cancer Research 1 (1): 41-46, 2005
ISSN 1811-9727

Algal Diets Reverse Diethyl Nitrosamine (DEN) Induced Hepatocarcinoma in Rats

[1]M. Harish Kumar, [1]K.S. Radha and [2]S.C. Gajaria
[1]Department of Physiology II, Miyazaki Medical College, Miyazaki, Japan
[2]Laboratory of Applied Phycology, Department of Biosciences, Sardar Patel University,
Vallabh Vidya Nagar 388 120, Gujarat, India

Abstract: We investigated the effects of algae (*Chlorella vulgaris* and *Enteromorpha* sp.) incorporated diets in the prevention of diethyl nitrosamine (DEN: C_4H_{10}-N_{2O}) induced hepatocarcinoma in rats. Toxicity was induced by i.p. administration of DEN in multiple doses @ 150 mg kg^{-1} BW. Rats were divided into 8 experimental Groups and fed either with control diet (synthetic diet without algae) or algae incorporated synthetic diets for a period of 8 weeks. DEN treated rats fed with synthetic diet showed a significant ($p<0.05\%$) reduction in bodyweight, organ weight and relative organ/bodyweight, while Groups fed with algal diets showed gradual recovery from the morphological phenomenon. Likewise, algal diets helped in the process of improvement from liver damage as judged by studying the fluctuation of liver enzymes namely cytochrome P-450, SGPT, G-6-phosphatase and amino-n-demethylase. Serum enzymes, cathepsin B, cathepsin D, ß-D-galactosidases and glucosidases showed significantly ($p<0.05\%$) lower activities in algal diet fed Groups than the synthetic diet fed Group. These results imply or suggest that a combination of *Chlorella* and *Enteromorpha* in diets is efficient in limiting the action of DEN during the initiation phase of hepatocarcinogenesis in rats and may help in prevention of liver damage.

Key words: *Chlorella vulgaris*, *Enteromorpha*, diethyl nitrosamine (DEN), liver enzymes, lysosomal enzymes

INTRODUCTION

Diethyl nitrosamine (DEN; C_4H_{10}-N_2O; MW 102.14), used as an additive in gasoline and lubricant oil and as a stabilizer compound in plastic industry is a known carcinogen. Nitrosocompound lead tumurogenesis, as described by Boremblunis theory, is a two stage process involving initiation and promotion stages like DNA damage, leakage and repair of nuclear DNA, finally leading to other types of carcinogenesis *in vivo* by other metabolic pathways[1]. The nutritional and therapeutic capacity of algae and vegetables as protective dietary factors against many types of cancers is well understood[2-4]. Algal metabolites like pigents, peptides and carbohydrates are principle agents to prevent nitrosocompound carcinogenesis[5] in animal studies. In Asian countries algae have been traditionally consumed along with daily food. *Chlorella vulgaris* and *Enteromorpha* sp. belong to family Chlorophyceae. The principal components of *Chlorella* like chlorophyll, ß-carotene, Chlorella Growth Factor (CGF), many vitamins, minerals as well as dietary fibers are reported to have health benefits. Recent investigations show *Chlorella* to be a potent detoxification agent[6], tumor suppressing agent[7], stress-induced apoptosis reducing agent in mice[8], gene expression agent in human growth hormone production[9], a beneficial antimetastatic immunopotention organism[10] and a good feed additive for marine herbivorous fish[11]. *Chlorella vulgaris* also helps in prevention of infection from *Listeria monocytogens*[12], hepatotoxicity and lipid peroxidation[13] and cures fibromyalgia syndrome[14]. *Enteromorpha* is a macroscopic fresh water alga, exhibiting suppressive effect on initiation and promotion phase of chemically induced mouse skin tumorogenesis[15]. Bioactivation and detoxification system plays an important role in carcinogenesis and are regulated by phase 1 and phase 2 enzymes like cyto-P450 and GST, respectively.

The present research was designed to study the effect of *Chlorella vulgaris* and *Enteromorpha* in combination and individually at different concentrations in treating DEN induced hepatotoxicity in rats. To our knowledge, the effect of combined algal diets on the actions of these enzymes to study the hepatotoxicities treatment is a less focused area. Furthermore, this study may help to understand the extent of interaction between algal diets in xenobiotic induced liver damage and its prevention in animal model.

Corresponding Author: M. Harish Kumar, Department of Physiology II, Miyazaki Medical College, Miyazaki, Japan
Tel: +81 985 851785 Fax: +81 985 857932 E-mail: hkumar@med.miyazaki-u.ac.jp

MATERIALS AND METHODS

Chemicals: DEN and enzyme substrates of P-nitrophenol derivatives were obtained from Siga Fine chemical Co., St. Louis, MO-USA. All other chemicals and reagents used were of analytical grade and manufactured by SDH chemicals, Mumbai, India.

Animals and Housing: Wister strain rats were obtained from local scientific suppliers (Ahmedabad, India) and housed in polypropylene cages with sterile, inert husk materials as bedding. Commercial feed (Hindustan Lever feed Ltd, Mumbai) and water free of contaminants were provided *ad libitum*. The experimental animals were maintained in a controlled environment with 25°C and 60±5% relative humidity with 12 h light/dark cycle and were subjected to one week acclimatization period.

Organism (Algae): *Enteromorpha* sp. collected from fresh water river, was transported immediately to the laboratory and cleared of sand stone particles. Algae was washed in tap water, rinsed in deionised water, shade dried and powdered using electric ball mill. *Chlorella vulgaris* was grown in the laboratory in bioreactors using Arnon and Arnold medium. Fresh cells were harvested by centrifugation, rinsed in deionised water, dried and stored at room temperature in glass containers.

Statistical analysis: Analysis of data are presented as mean±SD for 10 rats in each Group. Significant differences between mean values were determined by one-way analysis of variance (ANOVA) and separated by using Duncan's Multiple comparison test. Comparison of the observed values for statistically significant differences were set at $p<0.05\%$. The analysis was carried out with the aid of the Prism Graphpad 3.03 version statistical package.

Experimental design: Prior to start of the experiment, all animals were caged and acclimatized for one week period and fed with synthetic diet. In total, 80 rats with body weights ranging from 185±2.1 to 202±1.6 g were used in the experiment and were divided into 8 Groups. While 7 Groups were administrated with DEN (in saline) intraperitoneally, daily, at 150 mg kg^{-1} body weight for 7 days, Group 8 alone received saline injection instead of DEN. Synthetic diet feeding was continued up to the second week i.e. till the completion of DEN course. Group 1 fed with synthetic diet alone and Group 8 animals that did not receive DEN served as control Groups. Experimental diets were prepared with different doses of powdered algae incorporated with the synthetic diet. Group 2 and 3 received diets containing 25 and 50% *Chlorella* dry powder mixed with synthetic diet proportionally. Group 4 received mixed diets containing 25% *Chlorella*, 25% *Enteromorpha* and 50% synthetic diet. *Enteromorpha* dry powder mixed at the rate of 25 and 50% along with synthetic diet proportionally was fed to Groups 5 and 6, respectively. Group 7 received diet of 40% *Chlorella*, 40% *Enteromorpha* and 20% synthetic diet. This feeding schedule continued up to 8 weeks. Feeding trail was monitored according to the standards of animal experimentation and feed water consumption was recorded on a daily basis. Daily observation of body weight and mortality rate was recorded as mean values on a weekly basis.

After the experimental period, blood from the animals was collected by cardiac puncture and centrifuged at 5000 rpm for 15 min. The serum was stored at -70°C for further analysis. All surviving rats were sacrificed by ex sanguification from the abdominal aorta under light anesthetic condition at the end of 8 weeks. Liver was dissected and 5 g was homogenized in phosphate buffer saline (PBS 0.05M, pH 7.2) using polytron homogenizer. Microsomes were prepared from liver by differential centrifugation method[15] and resuspended in Tris-HCl buffer (0.5 M, pH 7.2) containing 0.5 M sucrose and 10% glycerol.

Cyctochrome P450[16], Serum glutamate pyruvate transaminase (SGPT)[17], glucose-6-phosphatase[18], aminopyrine-n-demethylase[19] and total protein [20]were determined according to published methods. Activities of serum glycosidases namely ß-D-galactosidases, ß-D-glucosidases[21], cathepsin B[22] and cathepsin D[23] were estimated according to standard methods.

RESULTS

Quantitative data of body weight changes during the experimental period is given in Table 1. Treatment with DEN (Group 1) showed a significant reduction in body weight at the end of 8 weeks. Group 2 also showed a significant reduction in body weight ($p<0.05\%$) corresponding to the initial week, while other Groups showed a marginal improvement/increase in body weight. It is interesting to note that Group 7 showed a significant ($p<0.05\%$) increase in mean body weight from 190±1.7 g to 203±1.7 g at the termination of the experiment. Increase in the body weight was also noticed in the Group 8 animals but did not show any significant fluctuations in between weeks (Table 1). Basic data on mortality, body weight, liver weight and relative body vs liver weight ratio was recorded. As indicated in Table 2, mortality rate was highest in Group 1, showing the severity of toxicity in the animals. Rate of mortality reduced gradually as

Table 1: Body weight changes (g) in 8 weeks

	Weeks								
Groups	0	1	2	3	4	5	6	7	8
1	186±3.6	185±2.1	180±8.9	172±3.1	168±1.8	165±1.7	160±2.2	172±7.0	168±1.7
2	180±4.7	182±1.8	181±3.5	188±1.4	175±3.1	178±1.7	180±1.5	182±2.3	175±0.7
3	183±5.1	185±1.2	183±1.7	180±1.9	176±7.3	179±3.9	183±1.3	189±3.5	192±3.5
4	183±4.6	180±3.1	175±3.3	179±3.9	189±3.9	192±3.7	190±3.0	189±1.4	193±3.9
5	175±3.1	180±2.9	178±3.8	181±3.7	182±3.1	182±3.9	183±3.6	187±1.7	190±6.3
6	182±4.9	185±2.3	188±2.7	190±2.8	193±2.4	190±2.7	188±1.0[a]	190±1.8	195±1.2[a]
7	187±3.2	190±1.7	192±1.7	189±2.4	195±1.7	198±1.6	200±1.3[ab]	202±1.4	203±1.7[ab]
8	187±5.1	185±1.0	193±3.0	192±1.8	189±1.6	200±1.3	203±1.7[a]	209±1.9	205±1.3[a]

Values represent mean ±SD of three independent experiments, each consisting of data pooled from 10 rats. Superscript a denotes a significance (p<0.05%) than Group 1 and b denotes significant than Group 1 and Group 6 (Duncan's Multiple Range Test)

Table 2: Mortality rate, body weight, liver weight and relative liver weight after eight weeks of experimental periods

Groups	Mortality rate (%)	Body weight (g)	Liver weight (g)	Relative liver weight g/100 g BW
1	70	185±2.1	8.5±0.7	4.5
2	30	182±1.8	8.3±1.1	4.6
3	20	185±1.2[a]	9.8±0.4	5.2
4	00	180±3.1	10.3±1.2[b]	5.4[bd]
5	10	180±2.9	9.8±1.3	5.4[bd]
6	10	185±2.3[a]	8.8±1.0	4.7
7	00[a]	190±1.7	10.3±1.9[bc]	5.7
8	00[ab]	202±1.6[ab]	12.1±0.9[bc]	6.3[bd]

Values represent mean±SD of three independent experiments, each involving 10 rats

a= Significant p<0.05% as compared to Group 1

b= Significant p<0.05% as compared to Group 1 and Group 6

c= Significant p<0.05% as compared to Group 1, Group 6 and Group 7

d= Significant p<0.05% as compared to Group1, Group 4, Group 5 and Group 8

Table 3: Activities of serum cathepsins and glycosidases

Groups	Cathepsin B	Cathepsin D	ß-D-galactosidase	ß-D-glucosidase
1	318±4.1[a]	410±2.8	31.4±1.5[a]	110±0.1[a]
2	375±2.2	518±1.5	49.2±2.5	147±0.1
3	292±0.4	325±0.1	31.2±2.5	89±0.2
4	250±0.6	375±0.3	24.4±4.6	92±3.7
5	200±3.9	285±4.5	18.2±0.2	95±1.1
6	300±3.5[b]	395±2.4	28.2±1.4	95±1.1
7	170±4.6[b]	200±3.5[b]	21.2±2.3[b]	55±2.8
8	173±6.3	205±1.9	19.7±1.9	48±1.0

Serum Cathepsin and Glycosidases activity in rats treated with different algal diets and control diets (without algae) in different Groups. Enzyme activity was measured according to standard methods.

Values represent mean±SD of three independent experiments, each consisting of tissues pooled from 10 rats. Cathepsin B unit: n mol P-nitroanilide liberated/h/mL serum. Cathepsin D unit: n mol of tyrosine formed/h/mL serum. ß-D-galactosidase unit: n mol p-nitrophenol liberated/h/mL serum. ß-D-glucosidases unit: n mol p-nitrophenol liberated/h/mL serum.

a= Significant at p<0.05% as compared to Group 1

b= Significant at p<0.05% as compared to Group1 and Group 6

c= Significant at p<0.05% as compared to Group 1 in Cathepsin –D activity. Cathepsin B activity showed significant as compared to Group 1 to Group 7. ß–D-Glucosidase activity showed Significant at p<0.05% as compared to Group 1 to Group 6

incorporation of algae in the diet increased. Pattern of liver weight changes reveals the lowest relative liver weight of 4.5/100 g BW (p<0.05%) in Group 1. On the other hand, all the other Groups, which were given algal powder, did show improvement in relative liver index. *Chlorella* and *Enteromorpha* at 40% w/w basis (Group 7)

Table 4: Activities of liver enzymes after 8 weeks after treating with DEN and algal diets

Groups	CytoP-450	SGPT	G-6-phosphatase	Amino-N-demethylase
1	1.1±0.1	53.8±0.2	205.1±1.7	10.8±0.1
2	0.6±0.1[a]	52.4±0.2[a]	175.2±0.09	2.6±0.3
3	0.7±0.1	24.5±0.3	162.8±1.5	0.9±0.1
4	0.9±0.1	27.5±0.2	185.3±2.6	9.5±0.2
5	0.5±0.2	32.4±0.1	85.4±0.9	3.7±0.2
6	0.9±0.3	37.5±0.4	200.1±1.0	7.9±0.8
7	0.4±0.2[ab]	29.4±0.2[ab]	198.4±1.4	9.9±0.2[ab]
8	0.5±0.3	32.6±4.7[c]	185.0±1.3	8.5±0.2

Values represent mean±SD of three independent experiments, each consisting of tissues pooled from 10 rats. Cytochrome-p450 unit: n mol mg^{-1} protein SGPT unit: Units/mL/mg protein; G6 phosphate unit: n mol P liberated/min/mg protein

Amino-N-demethylase unit: n mol/min/mg protein

a= Significant at p<0.05% as compared to Group 1

b= Significant at p<0.05% as compared to Group1 and Group 2

c= Significant at p<0.05% as compared to Group

showed the highest value of 5.7/100 g BW (Table 2). This result suggests that algae at highest concentration do have beneficiary effect on gross health condition of rats.

Serum enzymes: Table 3 summarizes the activities of the marker enzymes cathepsin B, cathepsin D, ß-D-galactosidase and ß-D-glucosidase in serum. A significant (p<0.05%) enhancement of all four enzymes were observed in DEN treated animals (Group 1). Notably, Group 7 fed with the highest concentration of algae in combination showed highly decreased activity that is statistically significant (p<0.05%) and almost similar to Group 8. Interestingly, ß-D-galactosidase alone was significantly higher (p<0.05%) in 25% Enteromorpha fed Group than the control values. ß-D-glucosidases in the rats given DEN (Group1) was significantly higher as compared to animals treated with algal diets and non treated animals (Group 8).

Liver enzymes: In order to explore the index of DEN toxicity and cancer prevention by algal diets, activities of different liver enzymes namely Cyto-P450, SGPT, G-6-phosphatase and amino-N-demethylase were measured (Table 4). SGPT activity in algal diet fed Groups tended to be lower than DEN treated animals (Group1). The elevated activity of Cyto-P450 in Group1 is also notable

observation in the present study. Algal diets significantly decreased the G-6-phosphatase activities than the synthetic diet. The elevated activity of Amino-N-demethylase activity induced by DEN was reduced significantly ($p<0.05\%$) in Groups 5 and 6, indicating that elevated enzyme activity induced by DEN can be suppressed to normal values by consumption of algal diets.

DISCUSSION

It is known for decades that consumption of algae and seaweed reduces the risk of many types of cancer in human beings. Glycosidases are lysosomal enzymes responsible for degradation of carbohydrate moieties of glycoprotein, lipid, glycosaminoglycans and are known marker enzymes in cancer study[24]. Likewise an elevated activity of cathepsin-a protease is also an index of malignancy[25,26].

Wattenburg[27] showed that the synergistic actions of blocking agents occurring in diets are important in cancer prevention. Morita *et al*.[6] found that dietary fiber in algae is a good agent for reduction of PCB and dioxin toxicity in rats. Dietary fibers are digested slowly in colon to form short chain fatty acids like acetate, propionate and butyrate by fermentation. It has been proposed that butyrate inhibits cell proliferation, promotes cell differentiation and leads to apoptosis, contributing to the healthy condition of colon mucosa[28]. This study, combination of chlorella with *Enteromorpha* at 40% concentration level showed efficiency in preventing DEN induced carcinogenesis. Chlorophyllin-a chlorophyll derivative forms a complex with heterocyclic amines[29,30]. Chlorella and *Enteromorpha* have rich amount of chlorophyll and fiber respectively, which form complex with DEN thereby reducing the toxicity. Another line of studies by Lipman *et al*.[31] showed that retinoid in vegetables and fruits decrease carcinogenicity by inhibiting the cytochrome P-450 directed metabolic product. In the present study, high cytochrome P-450 (cyto-P450) in Group 1 reduced gradually in algae incorporated diets and showed lowest activity of 0.5±0.08 in Group 7 (Table 4). Nutritional and non-nutritional factors have direct relationship on the expression of the highly inducible cyto-P450 activity in animals and humans. Cytochrome P-450 is known to catalyze the N-oxidation of 4-aminophenyle, 2-napthalamine and several aromatic amines, which have proved to increase the risks of cancer. It is a key enzyme in the pathway of heterocyclic amine metabolic activation leading to final DNA binding forms[1]. Levels of cytochrome P-450 in rat liver is modulated selectively by retinoids that scavenge the reactive oxygen [RO] in animal body. In agreeing with results of Lipmann *et al*.[31] this study shows that *Chlorella* and *Enteromorpha* with good amounts of total retinoids and chlorophyll are better for hepatotoxic prevention since they have higher amount of active compounds. The exact mechanism could not be drawn in this experiment, but, indirect interaction of algal metabolites like chlorophyll and retinoids and marker enzymes in animal body is likely the mechanism of actions in prevention of further metabolism of DEN in animal body. The present study implies that algal ingredients mainly ß-carotene and chlorophyllin block the carcinogen metabolic activation and enhance the carcinogen detoxification in animals. Bhat *et al*.[32] studied that phycocyanin prevents hepatotoxicity, by causing a significant decrease of G-6-phosphatase and amino-n-demethylase activities in carbon tetrachloride induced hepatotoxicity in liver. Similarly, we observed the elevated activity of these two enzymes in DEN treated animals (Table 4), be significantly reduced with the aid of the algal diets.

Infiltration and migration of tumor cells to surrounding tissues is facilitated and enhanced by destruction and modification of structural proteins by release of proteolytic enzymes that solubilise the extra cellular matrix[33]. Serum proteases and glycosidases have been proposed as potential indicator molecules of inflammation condition in human beings. It may be possible that differential fluctuation of protease glycosidase activity in serum could modify the integrity of cell surface as stated by Bosmann[34]. Elevated levels of cathepsins B and D is a significant observation in human breast cancer cells[13] and gastric cancer [28]. The mechanism of increase in lysosomal enzymes activity during the course of carcinogenesis is not completely clear but latent amount of activity can be liberated during injury of cells in body inflammation conditions. In our studies, wherein we examined the influence of algal diets on the activities of cathepsins B and D, we found a significant increase ($p<0.05\%$) in both the cathepsins in DEN treated Group than the untreated or algal diets treated Groups (Table 2). Toda and Yokogoshi's[35] suggestion that soya protein isolate caused an improvement in muscle condition through reduction of proteosome activities may indicate that in the current study, protein moiety present in the algae interacted with the cathepsins B and D to help in the detoxification of DEN to ameliorate the muscle atrophy, finally leading to improvement of body weight. It is probable that administration of *Chlorella* and *enteromorpha* in diets may prevent the migration of T-cell and ß cells as observed by Cheng *et al*.[36] either by decreasing the

protease activity or checking the onslaught of free radical attack and decreasing the augmented levels of cathepsins B and D and glycosidases in carcinogen induced animals. However specific cell line *in vitro* experiments are required to establish this hypothesis. Although results are promising, it is difficult to judge the pattern of action of algal active ingredients *in vivo* at present. Therefore, it is necessary to investigate the mechanisms of actions of other enzyme pathways and at the molecular level.

In conclusion, the present study reveals that active compounds present in *Chlorella* and *Enteromorpha* suppress cancer promotion is an interesting and promising subject for future study. Daily intake of algal diets such as *Chlorella* and *Enteromorpha* may be useful against hepatotoxicity.

ACKNOWLEDGMENT

The authors thank the Head of the Department for providing with facilities to carry out this study.

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International Journal of Cancer Research 1 (1): 47-52, 2005
ISSN 1811-9727

Correlation Between Thymidine Phosphorylase Expression and Sex of Patients in Colorectal Carcinoma

[1,2]Sepideh Arbabi Bidgoli, [3]Bagher Minaee, [2]Mohammad H. Ghahremani,
[4]Mansoor Djamali Zavarei, [2]Shamileh Fouladdel and [2]Ebrahim Azizi
[1]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Azad University of Medical Sciences,
[2]Molecular Research Laboratory, Department of Pharmacology and Toxicology, Faculty of Pharmacy,
[3]Department of Anatomy and Embryology, [4]Department of Pathology, Faculty of Medicine,
Tehran University of Medical Sciences, Tehran, Iran

Abstract: The present study was carried out to determine the TP status in colorectal cancer and to correlate molecular alterations with sex and other clinicopathological findings. Out of 129 surgically resected colorectal cancer patients, 51 tumor samples (23 males, 28 females) were randomly analyzed by immunohistochemical techniques using primary antibody for TP and LSAB2 detection kit. Despite the lack of significant correlation between patients' sex and most other clinicopathological parameters, the mean tumor size in males (5.7174±1.8453 cm) was significantly (p=0.016) more than females (4.4643±1.8453 cm) in this study. Out of 51 postoperative colorectal tumor samples, 24 (47.1%) showed positive TP expression. Unlike other clinicopathological parameters, TP immunostaining was significantly correlated with tumor size (p=0.007), lymphatic invasion (p= 0.036) and sex of patients (p=0.002). The prevalence of TP positive immunostaining was significantly higher in males than females (66.6% versus 33.3%, respectively). Due to the importance of high TP expression in predicting the tumor responses to fluoropyrimidines, the results of the present study possibly show the role of sex hormones in TP expression and angiogenesis. This finding might be important in being considered as a valuable prognostic or predictive marker in clinical settings.

Key words: Colorectal cancer, Thymidine Phosphorylase (TP), sex, angiogenesis

INTRODUCTION

Colorectal cancer is a commonly diagnosed cancer in both men and women. It is estimated that in 2004, 146,940 new cases would be diagnosed and 56,730 deaths from colorectal cancer would occur[1]. It is accepted that the standard regimen in colorectal cancer is the fluoropyrimidine-based chemotherapy and this group of drugs is the most commonly used class of drugs in the treatment of patients with colorectal malignancies[2,3]. Recent studies have shown that the angiogenic factor Thymidine Phosphorylase (TP), also known as platelet-derived endothelial cell growth factor, influences the survival of patients with colorectal tumors[4]. Compared with *in vitro* studies showing that an increased intracellular level of TP in tumor cells treated with 5-FU-based protocols potentiates the activity of 5-FU by converting it to the more cytotoxic nucleoside form, it was found that sometimes patients who had tumors with the higher basal TP expression levels did not respond to 5-FU-based chemotherapy[2,3]. Therefore, the therapeutic interest in TP falls into two categories: Firstly, there are drugs, which are the substrates for TP and for these compounds, enhancers of TP levels and gene delivery of TP, promotes their cytotoxic activities[5]. Since the expression of TP, is strongly associated with clinical outcome for colorectal cancer, the newer colorectal cancer chemotherapy should be targeted to TP[6]. Secondly, TP is identified as a major angiogenic factor which promotes angiogenesis and metastasis of tumors if it is linked to their angiogenic properties[7]. Therefore, the realization that the growth and spread of tumors are dependent on angiogenesis has created new avenues of research designed to help us to better understand cancer biology and to facilitate the development of new therapeutic strategies[8]. Of the identified angiogenic factors,

Corresponding Author: Dr. Ebrahim Azizi, Director of Molecular Research Laboratory,
Department of Pharmacology and Toxicology, Faculty of Pharmacy,
Tehran University of Medical Sciences, Tehran, P.O. Box 14155-6451, Iran
Tel/Fax: (+98-21) 695 9100 or 646 1178 E-mail: aziziebr@sina.tums.ac.ir

PD-ECGF/Thymidine Phosphorylase (TP) has been recognized as one of the most frequently associated proteins with tumor progression and metastasis[9,10].

On the other hand, microvascular endothelial cells express the estrogen receptor and estrogen stimulates endothelial cell proliferation[11]. Estrogen may also promote new blood vessel development by acting directly on microvascular endothelial cells. It is proposed in endometrium that estrogen, by regulating expression and secretion of angiogenic factors by glandular epithelial cells, regulates endometrial angiogenesis[12] .

Nevertheless, it is not clear in the colorectal cancer whether estrogen promotes angiogenesis directly and/or indirectly via expression of angiogenic factors by particular colorectal glandular epithelial cells. As the identification of high TP expression may allow us to understand colorectal tumour biology and to predict the chemotherapy response to fluoropyrimidines with respect to sex of patients and sexual hormones and to use alternative therapeutic strategies, we aimed in the present study to evaluate the importance of this angiogenic factor as prognostic marker and its correlation with sex of patients in a clinical setting.

MATERIALS AND METHODS

Patients' characteristics: The questionnaires of 129 colorectal cancer patients were completed in three different university hospitals of Tehran (Imam Hossein, Shohadaye Tajrish and Imam Khomeini) during the years 2000-2003. Based on questionnaire, patients with prior chemotherapy or radiotherapy before surgery, familial history of colorectal cancer, history of background diseases and/or history of addiction to alcohol and abuse-substances were excluded from immunohistochemical study. Then, tissue samples of 51 patients with colorectal adenocarcinoma were randomly selected for the study. All of included patients were Iranian from different geographical locations within Iran. Based on the designed questionnaire, data were collected for their age at surgery, sex and pathological diagnosis. Histopathological data contained tumor anatomical location, tumor pathological type, tumor size, histological differentiation (malignancy grade), stage and lymphatic invasion.

TP immunohistochemical analyses: As previously described[13], dewaxed and rehydrated tissue sections were subjected to antigen retrieval using microwave oven and boiling citrate buffer (pH = 6.0). Endogenous peroxidase activity and nonspecific binding sites were blocked by incubating sections in 3% hydrogen peroxide in methanol for 30 min and 5% BSA for 60 min, respectively. Sections were then incubated overnight at 4°C with TP mouse monoclonal antibody (P-GF.44C, LabVision Corporation) that recognizes nuclear and cytoplasmic expression of the human TP protein. The primary antibody was used at dilution of 1:100. The results were visualized using the Streptavidine-biotin immunoperoxidase detection kit (LSAB2; Dakocytomation-Denmark) and DAB chromogen (Dakocytomation-Denmark) based on the manufacturer's instruction with necessary modifications. Sections were also counterstained with Meyer's haematoxyline. In each series, a section in which incubation with the primary antibody was omitted used as negative control. The ideal staining conditions were established in our preliminary experiments. Staining was considered negative only after careful examination of the entire tissue section. Quantitation of the intensity and number of positive tumor cells was performed by two independent pathologists (B. M. and M. D.) blinded to the clinical outcome. In cases in which the observers disagreed, the immunohistochemical scoring was repeated to agree on same scoring by both observers. Tumor samples were then classified into four categories based on the cytoplasmic expression of TP. Tumor cells were scored as 3+ if they had strong cytoplasmic staining (>50%), 2+ if they had moderate cytoplasmic staining (25-50%), 1+ if they had mild cytoplasmic staining (5-25%) and 0 if staining was <5% or no staining. According to the guidelines for pathological studies on colorectal carcinoma histological grade and pathological stage of tumors were also determined[14].

Statistical analyses: For the statistical analyses, descriptive data were expressed as the mean±SD. To compare means of continuous variables, independent sample T test, to compare discontinuous variables Man Whitney U test and to determine the correlation between molecular features and clinicopathological parameters the spearman's correlation test were performed using SPSS12 software[15]. The correlation between scores of TP expression and tumor anatomical location, size, histological differentiation, lymphatic and secondary organ metastasis, stage, as well as patients' sex and age at surgery were statistically evaluated.

RESULTS

Patients' characteristics: This study included 129 postoperative colorectal carcinoma, 69 (53.5%) males and 60 (46.5%) females. The tumor anatomical location was 60 (46.5%) in colon and 69(53.5%) in rectum. The mean

Table 1: Clinicopathological features of colorectal cancer patients

Age	54.4 year±15.28	Tumor size	5±1.84 cm
(mean±SD)	(range: 15-85)	(mean±SD)	(range: 0.5-9)
Sex		Lymphatic invasion	
Male	23 (45.1%)	Positive	24 (47.1%)
Female	28 (54.9%)	Negative	27 (52.9%)
Tumor anatomical level		Duke stages	
Colon	32 (62.7%)	Stage A	3 (5.9%)
Rectum	19(37.3%)	Stage B1	2(3.9%)
Histological Grade			
I	23 (45.1%)	Stage B2	22(43.1%)
II	22 (43.1%)	Stage C1	2(3.9%)
III	3 (5.9%)	Stage C2	10(19.6%)
IV	3 (5.9%)	Stage D	12(23.5%)
Secondary organ metastasis			
Positive	16 (31.4%)		
Negative	35 (68.6%)		

Table 2: Correlation between sex and clinicopathological features of patients

Variables	Males (n=23)	Females (n=28)	p-value
Mean of age	58.2±12.6	52.1±16.2	0.137
Tumor anatomical level			
Colon	12	20	0.161
Rectum	11	8	
Histological grade			
I	9	14	0.655
II	12	10	
III	0	3	
IV	2	1	
Tumor pathological stage			
A	0	3	0.117
B1	1	1	
B2	9	13	
C1	1	1	
C2	5	5	
D	7	5	
Lymphatic invasion			
Positive	13	11	0.228
Negative	10	17	
Mean of tumor size	5.7±1.81	4.47±1.7	0.014*
Secondary organ metastasis			
Positive	10	6	0.095
Negative	13	22	

* = Significant

age of these patients was 55.6 year±13.31 (range: 15-85). No significant difference were observed between the mean age of females 54.91±14.3 when compared to the mean age of males 56.42±12.5 (p=0.525).

Clinicopathological features: As described in materials and methods, 51 patients with lack of prior chemotherapy or radiotherapy before surgery, familial history of colorectal cancer, history of background diseases and/or history of addiction to alcohol and abuse-substances were selected for immunohistochemical studies. In randomly selected patients the distribution of tumor anatomical level was 32 (62.7%) in colon and 19 (37.3%) in the rectum. The mean macroscopic size of the resected tumors was 5 cm (range: 0.5-9). The carcinomas were 23 (45.1%) well differentiated, 22 (43.1%) moderately differentiated, 3 (5.95%) poorly differentiated and 3 (5.9%) undifferentiated. More than half of the samples 27 (52.9%) were also lymph node negative and 24 (47.1%) of these samples had positive lymphatic invasion (Table 1). Among these cases, 16 patients (31.4%) had also secondary organ metastasis.

Relationships between sex and clinicopathological features of patients: Despite the lack of significant difference between patients' sex and age (p=0.137), tumor anatomical level (p=0.161), histological grade (p=0.655), tumor pathological stage (p=0.117), lymphatic invasion (p=0.228) and secondary organ metastasis (p=0.095) (Table 2) we observed a highly significant difference between males and females with respect to tumor size (p=0.014). The mean tumor size of colorectal tumors in males (5.72±1.84 cm) were significantly higher than females (4.46±1.85 cm).

Results of immunostaining with TP antibody: Out of 51 postoperative colorectal cancer patients, 24 (47.1%) showed TP expression. The distribution of positive

Table 3: Clinicopathological significance of TP (+/-) cases of colorectal cancer

Variables	Negative	1(+)	2(+)	3(+)	p-value
Age					
>54	15	6	4	0	0.366
<54	12	9	4	1	
Sex					
Male	7	9	6	1	0.002**
Female	20	6	2	0	
Tumor location					
Colon	18	10	3	1	0.402
Rectum	9	5	5	0	
Histological grade					
I	14	7	3	0	0.405
II	11	6	4	1	
III	1	1	1	0	
IV	1	0	0	0	
Tumor size					0.007**
>5 cm	11	10	7	1	
<5 cm	16	5	1	0	
Lymphatic invasion					
Positive	11	10	7	1	0.036*
Negative	16	5	3	0	
Secondary organ metastasis					
Positive	8	6	2	0	0.982
Negative	19	9	6	1	
Pathological stage					
A	3	0	0	0	0.276
B1	0	1	1	0	
B2	14	6	2	0	
C1	1	0	1	0	
C2	3	3	3	1	
D	6	5	1	0	

*, ** Represent significant and highly significant, respectively

staining for TP was 1 (2%) with strong staining (3+), 8 (15.7%) with moderate staining (2+) and 15 (29.4%) with mild staining (1+).

Relationship between TP expression and clinicopathological findings: Based on the results of IHC staining, the percent of patients did not differ significantly between negative and positive TP with respect to age (p=0.336), tumor location (p=0.402), histological differentiation (p=0.405), secondary organ metastasis (p=0.982) and pathological stage (p=0.276). Unlike mentioned parameters, TP immunostaining was significantly correlated with tumor size (p=0.007). The prevalence of TP negative staining was significantly higher in tumors with <5 cm of size when compared with tumor size >5 cm (59% versus 41%, respectively). The prevalence of TP positive staining was significantly higher in tumors with size >5 cm when compared with <5 cm (75% versus 25%, respectively) (Fig. 1).

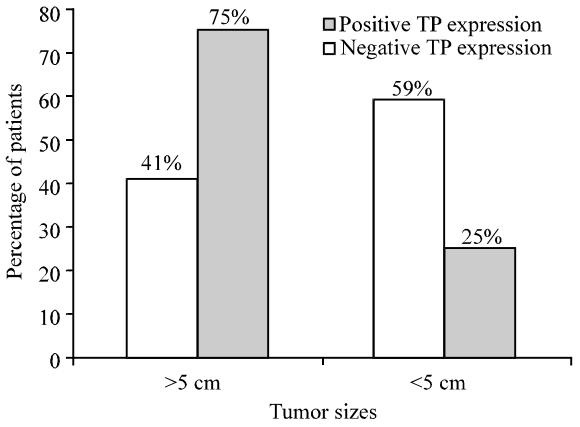

Fig. 1: Status of TP expression with respect to tumor size. The prevalence of TP negative staining was significantly higher in tumors with <5 cm of size when compared with >5 cm of size. The prevalence of TP positive staining was significantly higher in tumors with size >5 cm when compared with <5 cm

TP immunostainings were also correlated with sex of patients (p=0.002) (Table 3). The prevalence of TP negative immunostaining was significantly higher in females than males (74% versus 26%, respectively) and the prevalence of TP positive staining was significantly

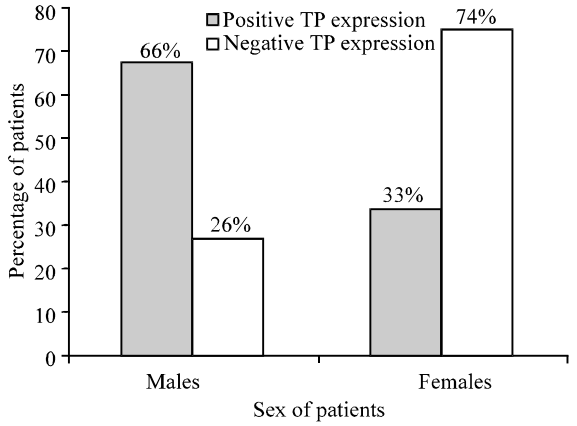

Fig. 2: Status of TP expression with respect to sex. The prevalence of TP negative immunostaining was significantly higher in females than males. The prevalence of TP positive staining was significantly higher in males than females

higher in males than females (66.6% versus 33.3%, respectively) (Fig. 2). A significant correlation were observed between TP expression and lymphatic invasion (p=0.036) (Table 3). Out of 29 lymph node positive patients, 18 (62%) had positive TP staining as opposed to 8 patients (38%) in 24 lymph node negative patients.

DISCUSSION

Colorectal cancer represents one of the best-studied models of tumor angiogenesis. A large number of angiogenic factors have been identified that regulate angiogenesis in colon cancer, including VEGF, b-FGF, TP/PD-ECGF, IL-8, Ang-1, Ang-2, TGF-$, TNF-" and angiogenin[8,16,17]. Among these factors, VEGF and TP/PD-ECGF are the most closely associated with tumor angiogenesis and progression in colon cancer[9]. Analysis of primary human malignancies, including colon, esophageal, gastric, breast, bladder, ovarian and lung cancers showed that TP expression was elevated up to 10 fold in the tumors when compared with the corresponding non-neoplastic regions of the same organs[18]. In the majority of these studies, higher TP expression was linked to increases in angiogenesis, invasion, metastasis and to shorter patient survival[18]. In the present study, close association between TP expression and lymphatic invasion was observed (p=0.036). In studies of GI cancers in which multiple angiogenic factors were examined, TP was found to be an independent prognostic marker of tumor aggressiveness[19,20]. Takahashi *et al.* [21] hypothesized that TP/PD-ECGF drives angiogenesis in colon tumors. Present results showed the correlation between size of colorectal tumors and positive expression of TP/PD-ECGF (p=0.007). It is clear that the growth and spread of tumors are dependent on angiogenesis which is a new avenue of research and potential therapeutic opportunities[8]. A highly significant difference between males and females with respect to tumor size (p=0.016) were observed in our study. The mean tumor size of colorectal tumors in males was significantly more than females. This result indicates the possible different angiogenic inducers in males and females, which could be affected by the sex hormones. However, there is no data about the role of sex hormones on angiogenic process in colorectal cancer. Aoki *et al.*[22] have recently reported that estradiol could induce the expression of TP/PD-ECGF and its mRNA and tamoxifen greatly could diminish the estrogen-induced TP/PD-ECGF in uterine endometrial cancer cells. They reported that the estrogen-induced TP/PD-ECGF might be driven via estrogen receptor cascade. The suggested estrogen-induced TP/PD-ECGF expression was increased approximately two-fold by progesterone and by its metabolite 17 alpha hydroxyprogesterone in well-differentiated endometrial cancer cells, but not with other various steroid hormones[22]. Although we observed a possible negative role of female sex hormones on TP/PD-ECGF expression and tumor size in colorectal tumors but the mean age of female cases were showed their menopause phase of life. Furthermore, the role of male sex hormones is not even understood in relation with angiogenesis. It seems that male hormones can induce TP expression in colorectal carcinoma with larger size.

There is no specific study on the role of sex on angiogenesis and TP expression and optimization of chemotherapy regimen with respect to the sex of patients as well as other factors. Present data suggest the possible role of sex hormones on colorectal tumor growth. Due to significance of high TP expression in predicting the chemotherapy response to fluoropyrimidines[23] and present findings on correlation of TP and sex, it seems to be important to consider the role of sex in choosing chemotherapy regimen for patients.

ACKNOWLEDGMENTS

Authors are thankful to the office of Vice-Chancellor for Research of Tehran University of Medical Sciences (TUMS) for their financial support of this research project. We also would like to thank Dr. I. Jahanzad and Dr. M.R. Khoshayand for their advices on IHC studies and statistical analysis, respectively. We appreciate sincere cooperation of the staff of Imam Hossein,

Shohadaye-Tajrish and Imam Khomeini University Hospitals on providing tumor samples.

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International Journal of Cancer Research 1 (1): 53-56, 2005
ISSN 1811-9727

Effects of an Antioxidant Extract on Adenosine Deaminase Activities in Cancerous Human Liver Tissues

[1]A. Avci, [2]M. Kaçmaz, [3]M. Kavutcu, [4]E. Göçmen and [1]¤. Durak
[1]Department of Biochemistry, School of Medicine, Ankara University, Ankara, Turkey
[2]Department of Biochemistry, Faculty of Medical, K?r?kkale University, K?r?kkale, Turkey
[3]Department of Biochemistry, Faculty of Medical, Gazi University, Ankara, Turkey
[4]General Surgery Clinics, Numune Hospital, Ankara, Turkey

Abstract: Effects of an antioxidant extract consisting mainly of garlic (*Allium sativum*) and red clover (*Trifolium pratense*) on adenosine deaminase (ADA) activities were investigated in cancerous and non cancerous human liver tissues and, results were compared with those of fludarabine. Ten cancerous and 10 non cancerous adjacent liver tissues were obtained from patients with metastatic type liver cancer by surgical operations. Kinetic analyses were carried out to establish V_{max} and K_m values of the reaction catalyzed by ADA under normal and inhibitor conditions. ADA activity was found lower in the cancerous tissues compared with non cancerous ones. Antioxidant extract created significant inhibitions on the ADA activities both in cancerous and non cancerous tissues. Inhibition percents were relatively higher in the cancerous tissues as compared with non cancerous ones. Furthermore, we have observed that inhibition percents created by the extract are higher than those of fludarabine at the concentrations studied. Results suggest that antioxidant extract exerts significant inhibition on the ADA activity in cancerous and non-cancerous human liver tissues. This might be rational basis for the use of these herbs in the alternative cancer therapy in the folk medicine.

Key words: Cancer, adenosine deaminase, antioxidant, garlic, red clover

INTRODUCTION

Adenosine deaminase is an important enzyme in the degradation of adenine nucleotides[1]. It is accepted as a key enzyme in purine metabolism and DNA turn over and thus, in the cancer process[2,3]. In several studies, tissue ADA activities were found increased, decreased or unchanged depending on types of tissue and cells studied[4-8].

Fludarabine (fludarabine phosphate) is a chemopreventive used in thetherapy of several types of cancers[9,10]. Its chemotherapeutic potential is known to be mainly resulted from its inhibitory activity on the ADA enzyme[10-12].

Epidemiological evidence has revealed that garlic consumption has played a significant role in the reduction of deaths caused by malignant diseases[13,14]. For example, it has been reported that the mortality rate among patients with gastric cancer was significantly lower in regions of China with higher garlic consumption[13]. Similar beneficial effects of garlic were observed in cases of gastric cancer in Italy[15]. It has been found that garlic consumption has led to decreased incidence of colorectal cancer among Japanese[16]. Alliin and one of its metabolites, allicin, were found to show tumor inhibiting effects[17,18]. Since then, although a number of studies have been made on the antitumor and cytotoxic actions of garlic and its organosulfure constituents[19-24], precise mechanism(s) on the anticarcinogenic action of garlic has not been clarified yet.

Similar to the garlic, red clover has long been used in the alternative cancer therapy among people. It is accepted to be one of the world's oldest and most common natural cancer remedies[25]. Although its anticancer compounds (isoflavones like genistein) may make it an effective anticancer food[26,27], the scientific study on red clover is still new and further researches are needed to clarify its possible role and action mechanism in the cancer treatment.

Corresponding Author: Dr. ¤lker Durak, Professor, Ankara Üniversitesi T?p Fakültesi,
Biyokimya Anabilim Dal? (Dekanl?k Binas?), 06100 Sihhiye, Ankara, Turkey
Tel: +90 312 3116457 Fax: +90 312 3106370 E-mail: durak@medicine.ankara.edu.tr

MATERIALS AND METHODS

In the present study, 10 cancerous and 10 non cancerous adjacent liver tissues were obtained from 10 patients with metastatic type liver cancer originating from colon. After removed by surgical operation, tissues were washed with deionized water and stored at -30°C for about a month. Before the activity measurement, tissues were cleaned from the fatty part, homogenised in pH 7.2, 50 mM phosphate buffer and centrifuged at 10 000 rpm for 30 min. Upper clear layer was removed and used in the assays. ADA activity and protein amount were measured as described, respectively[28,29]. Kinetic analyses were carried out by incubating samples (100 µL) with extracts (10 µL) and with fludarabine (10 µL from -12.5 mg fludarabine phosphate/mL deionised water-solution).

Antioxidant extract was prepared by incubating 100 g fresh garlic and 10 g dry red clover in 500 mL water-alcohol-olive oil solution (100/10/1 v/v/v, respectively) for 10 days (SARMEX^R). After homogenisation, it was centrifuged at 10 000 rpm for 30 min and upper clear layer was removed to be used in the kinetic assays.

RESULTS AND DISCUSSION

ADA activity was lower in the cancerous liver tissue as compared with non cancerous adjacent one (Table 1). K_m value was also lower in the cancerous tissue, which was an indication for high affinity of the enzyme of the cancerous tissue against its substrate, namely adenosine.

Antioxidant extract and fludarabine both exerted significant inhibitions on the ADA activity but, inhibition percents were higher for antioxidant extract compared with fludarabine at the concentrations studied. Inhibition percents in the non cancerous tissue were 85% for antioxidant extract and 35% for fludarabine. The same inhibitions in the cancerous tissue were however 92% for the extract and 24% for fludarabine. K_m values calculated under inhibitor conditions were lower than those calculated without the extract and fludarabine.

Kinetic analyses showed mixed type inhibitions (Lowered V_{max} and K_m values for the inhibitors studied).

Garlic[30,31] and of a lesser degree red clover[32] are perhaps most widely quoted herbs with therapeutic potentials. In addition to several diseases like atherosclerosis, both have long been used as folk medicines in cancer therapy. Several epidemiological studies have revealed that garlic consumption is associated with reduced mortality and morbidity[15,16,33]. Some organosulfur compounds like alliin, allicin, s-allyl cysteine etc. have been accepted to play major role in this protective function[34-37]. Despite all these findings, no exact mechanism(s) and active component(s) in the garlic extract have not been clarified.

Red clover has also long been used to treat cancer and acoustic tumors among people. The use of red clover as an anticancer agent can be traced back to the 1940s when herbalist Harry Hoxey was promoting the herbs an alternative to surgery and radiation therapy[25,26,32]. Red clover contains high amounts of isoflavone compounds such as genistei[26]. Several researchers have shown that these isoflavones may help to prevent cancer[27]. In a study, it has been demonstrated that isoflavone derivatives inhibit the cell growth of stomach cancer lines *in vitro*[27] and supposed that this might occur through activation of a signal transduction pathway for apoptosis. In another, biochanin A, one of the isoflavones in red clover was found to inhibit carcinogen activation in cells in culture medium[27]. However, the precise mechanism of action and responsible constituents for proposed benefits of red clover in the cancer process is unknown.

Fludarabine has been established to exert significant inhibition to the metabolic conversions of purines to their active triphosphates by ADA. In particular, it exhibits substantial activity against lymphoid malignancies such as chronic lymphocytic leukemia and non-Hodgkin's lymphoma[4]. Inhibition of ADA by purine nucleoside analogs such as fludarabine is the rational basis for use of these analogs in the cancer therapy.

Table 1: Mean±SD of adenosine deaminase (ADA) enzyme activities (V_{max} = mIU mg^{-1} protein) and K_m values (µM) in cancerous and non-cancerous human liver tissues measured in normal and inhibition conditions

	Control (n=10)		Cancer (n=10)		Student's t-test [Control vs. Cancer]	
Groups	V_{max} (mIU mg^{-1})	K_m (µM)	V_{max} (mIU mg^{-1})	K_m (µM)	V_{max}	K_m
A (without inhibitor)	3.99±1.33	145±46	3.08±1.24	120±35	p<0.050	p<0.05
B (with extract)	0.78±0.54	45±30	0.38±0.26	52±28	p<0.025	NS
C (with fludarabine)	2.61±1.52	100±26	2.36±1.10	70±20	NS	p<0.05
Student's t-test						
A vs. B	p<0.0005	p<0.0005	p<0.0005	p<0.0005	-	
A vs. C	p<0.05	p<0.01	p<0.05	p<0.005	-	
B vs. C	p<0.005	p<0.01	p<0.0005	p<0.05	-	

Under the lights of these explanations, how can our results be evaluated? We think that lowered ADA activity might be an attempt to slow rapid growth of cancer cells in the cancerous tissues. As seen from the Table 1, inhibition percents of the antioxidant extract in the cancerous and non-cancerous tissues are substantially higher than that of fludarabine at the concentrations studied. This finding may be one of the rational bases for the use of both herbs in the cancer therapy in the folk medicine. As far as we know, there is no study aiming to investigate possible effects of this kind of extracts on ADA activity in cancerous tissues. However, only in one study, it has been established that ADA is inhibited in aortic endothelial cells by garlic extract and suggested to contribute to the hypotensive activity and vessel protective effects of garlic[38]. Although there are several hypothetical explanations on how garlic and red clover extracts play part n the cancer preventive events, none has given satisfactory explanation yet. For example, it has been reported that organosulphur components might inhibit several tumor progressions in experimental animals[34,36,37]. However, action mechanisms of these compounds have not been clarified. Similarly, as to the anticancer potential of red clover, it has been reported that, an isoflavone from red clover can inhibit carcinogen activation in cells[27] and another isoflavone namely, biochanin A can inhibit tumor growth in stomach cancer cell lines[26]. However, activity mechanism of these compounds have not been documented yet. Lowered V_{max} and K_m values which is an indication of high affinity of the enzyme against its substrate under inhibition conditions reveal mixed type inhibition mechanisms for the substances used in the present study.

Results suggest that the constituents of the antioxidant extract cause significant inhibitions on ADA activity. It might be one of the rational basis for the use of these herbs in the alternative cancer therapy in folk medicine.

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International Journal of Cancer Research 1 (1): 57-59, 2005
ISSN 1811-9727

Esophageal Cancer in an Iranian 20 Years Old Young Male-A Case Report

Sh. Semnani, N. Abdollahi, Kh. Kalavi and R. Azarhoosh
University of Golestan Province Medical Sciences, Gorgan, Iran

Abstract: Present study introduces a case of esophagus cancer, who was a 20 years old young male Turkmen. He had been attended the polyclinics of 5-azar educative hospital in Gorgan, the capital city of Golestan province, located in north east of Iran. The patient had faced with a serious dysphagia with a progressive mode in nature, when attended. He had been experiencing the problem three months before attending. Because the high frequency of the esophagus cancer in the region, as expected, we found a papiloadenomatous mass along middle third of the esophagus duct, endoscopically. The diagnosis was epithelial squamous cell carcinoma of the esophagus due to pathologist's report. Unfortunately, the patient then, died in the operating room due to cardiovascular arrest.

Key words: Esophagus, young, elderly, Iran, north region, Trukmen

INTRODUCTION

The esophagus cancer is one of the most worst prognostic malignancies in human, that occur most commonly in adult individuals over 50 years old. The rate of its frequency among men to women in various populations varies from 2:1 to 20:1 and its incidence is about 1.5% of whole and 6% of GIS associated cancers, in USA[1]. The disease is the third one among common GIS involved malignancies and is one of the 10 common ones worldwide. It has been documented that the disease's occurrence varies in different populations probably due to geographic and cultural diversities that can be indicative of the possible environmental etiologic effects[2].

The occurrence of the esophagus cancer is more common in 6th, 7th and 8th decades of life; therefore, it is extremely rare during youth and childhood[3]. A few numbers of young and children with esophagus cancer have been reported yet[4]. Because of the long-term effects of environmental carcinogens, these factors may act as some minimal effectors on the disease etiology in young individuals; so its etiology remained still unknown[5].

The esophagus cancer occurs in two different forms, histologically:

1. The epithelial squamous cell carcinoma of the esophagus
2. The adenocarcinoma of the esophagus[1].

Almost 60% of epithelial cell carcinoma types occur in the middle and about 30% in distal and about 10% of the rest in the proximal third of the esophagus tube[1]. Five years survival rate of the disease had been about 1% among the blacks, 4% in whites; while according to developments in treatment trends, its rates increased to 9 and 13% in blacks and whites, respectively[6].

The incidence of the disease in USA is about 1000 cases/year; but it is more common in South Africa, Iran, china and India. Its frequency in north of Iran nearby Caspian seaside, is 206/100000 cases per year in men and 263 cases/100000 in women[7].

The etiology of the epithelial cell carcinoma of the esophagus is still unclear; but due to epidemiologic studies performed all over the world, there are strong associations between the onset of the disease and materials found in solid and liquid based food, water, tobacco, alcohol, nitrous amines, Candida infections, aflatoxines, avitaminosis especially riboflavin and selenium deficiency states. Other risk factors as obesity, low fruit and vegetable diets, hot beverages, asbestos and other cellular- molecular factors like oncogenes and tumor suppressor genes have been mentioned, too[7].

History: The patient was a young single male Turkmen aged 20 years old from Ag-gala one of the golestan province dependent cities. He had been attended the policlinics of 5-Azar educative hospital (GI specialist ward) in Gorgan, suffering from progressive dysphagia. There had been no evidence of clinical disturbances until three months prior to attending the ward; therefore, the clinical symptoms had initiated from that time with dyphagia against solids, even problems with fluids that had been associated with massive weight loss (10-15 kg). There were no symptoms of epigastric pain, nausea, vomiting, heart burn, hematemesis and melena at that

Corresponding Author: Kh. Kalavi, University of Golestan Province Medical Sciences, Gorgan, Iran
E-mail: kalavi20@yahoo.com Tel: 0098 171 4421651-53, 3351080

time. The patient's history showed no evidence of clinical problems before the occurrence of dysphagia. He had been a smoker since five years ago and opium addicted in smoking and oral mode, since five years ago. There were no symptoms of cervical lymphadenopathy and hepatomegaly, except for cachexia, when attended. Lab-tests showed: occult blood (negative), blood and hepatic function tests were all in normal ranges. Ultrasonography revealed no bulky and massive organic changes in liver, spleen, pancreas para aortal spaces, hepatic cord, gallbladder and bile ducts.

Considering dysphagia, a papiloma-adenematous mass defined endoscopically and diagnosis of a kind of infiltrative tumor cells of atypical epithelial squamous cell carcinoma of the esophagus confirmed, histologically (urease negative-sample). The patient, then confined to bed in the surgical ward to be esophagectomy, where unfortunately expired do to cardio vascular arrest in the operating room.

DISCUSSION

The esophagus cancer is rare in young (in less than 30 years old individuals)[3]. Based on a study (1952-1956), only three deaths (in <14 years) of esophagus cancer had been reported by US researchers[8]; as well there had been no such cases considering the two years survival rate (1969-1971) and SEER study programmes (1976). Moor *et al*.[9] have reported a fourteen years old boy with primary esophagus; however, details of the patient's conditions not reported. Kinnman *et al*.[10] have reported a case of fifteen years old male (well-differentiated type of esophagus cancer) with severe dysphagia. He had a history of lye ingestion when he was only three years old. The youngest patient had been an Indian girl (8 years old) that manifested the problem in middle third of her esophagus associated with lung metastasis[11]. Shahi *et al*.[11] have reported another Indian case (well-differentiated type) that was a 14 years old male. Abdullah *et al*.[12] had reported a 15 years old girl in 2000. This case that had contracted with SCC type, showed no relapse following esophagectomy until to end of that year. Dessueault *et al*.[13] have reported a 31 years old woman with psychogenic self-inductive vomiting that had SCC and Barrett's esophagus. Cheng *et al*.[14] have reported a 19 years old young with a history of dysphagia and postprandial regurgitation. This patient has had diffused esophageal liomyomatosis; so according to this, they have strongly suggested that in younger patients with a history of continuous dysphagia, one should consider the probable occurrence of liomyomatosis. Duvall *et al*.[15] have firstly reported as a case of adenocarcinoma of the esophagus in a young male who suffered from Cornelia de Lange syndrome with a history of reflux duration. Shah *et al*.[16] have introduced a case who was a Youngman suffering from a periodic episodes (month interval) of dysphagia and normal endoscopic close to the esophagus duct, which its resection showed a carcinoma completely fill an esophageal diverticulum's with a normal esophageal lumen. Dewer *et al*.[17] had reported a case of esophagus cancer in a young girl (20years old). In their case, the carcinoma of the esophagus had occurred because of osteosarcoma chemotherapy. Bolufendis *et al*.[18] have introduced a 31 years old male with a thirty days prolongation of dysphagia and weight loss, who had contracted with malignant melanoma. The patient then underwent a surgical operation of partial esophagectomy; but a month later, expired due to brain metastasis of tumor cells. Levin *et al*.[19] have reported six cases of esophagus cancer that the youngest one was a 26 years old patient. No one of them showed any significant and confidential clinical findings, in physical examination. Four cases had reported to have polyptoid masses in cardia and in all cases, tumor cells had involved distal areas and in five of six, there was a rate of dysphagia, when attended the ward. The illness is worse prognostic in the younger patients than elderly are[20-22] because:

1) Aggressive biologic nature of the disease among the young, 2) clinical and diagnostic presumption is not considered as an awful prognostic cancer among this group of patients.

According to a study established in Japan, there have been significant associations between prognostic state and types of mutations[23]. Regarding to some reports in young in comparison with elderly, there were no significant associations, considering sex, smoking and alcohol misuse and rate of death during surgical operation and prognosis[24-26]; therefore it has suggested that in young patients with continuous symptoms, endoscopic evaluations should be considered[24].

CONCLUSIONS

The esophageal cancer is a common problem in north region of Iran. Its frequency was also on an increasingly manner among young people. Therefore, it is strongly recommended that all cases especially younger ones referring policlinics and are going to be visited by physicians everywhere, should be examined more carefully and performed all available diagnostic methods to rule out the disease. Especially if manifestations of symptoms like dysphagia, reflux, Barrett's syndrome and other, doubtful GIS associated problems are present.

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International Journal of Cancer Research 1 (1): 60-70, 2005
ISSN 1811-9727

Polyphenolic Phytochemicals as Colorectal Cancer Chemopreventive Agents: An Intelligent Alternative to NSAIDs?

S. Sale, R.G. Tunstall and G. Garcea
Department of Surgery, The Leicester General Hospital, Leicester, LE5 4PW

Abstract: To review the evidence for using of polyphenolic phytochemicals as cancer chemopreventive agents. A literature search was undertaken from 1963 to the present day, using Medline and Pubmed. Epidemiological data suggests that diets rich in polyphenols confer significant protection against the risk of colorectal cancer. These observations are supported by *in vitro* and *in vivo* data. Polyphenols are polymechanistic in their anti-cancer action, but a common mechanism to all is their anti-oxidant property. Poor bioavailability, however, is potentially a limiting factor to their further development as clinical agents. Data regarding the chemopreventive efficacy of polyphenolic compounds is growing. Clinical trial data, although limited, suggests that such compounds show great potential for use as chemopreventive agents. The poor bioavailibity of polyphenols could be advantageous in achieving a localised therapeutic effect in the gastrointestinal tract, thus minimising the risk of unwanted effects in organs distant from the locus of absorption.

Key words: Polyphenols, colorectal cancer, chemoprevention

INTRODUCTION

Colorectal cancer is the second most common cause of cancer death in the western world. Efforts to reduce mortality from this disease are currently focussed on early detection of precursor lesions and polyps and early diagnosis of established cancers. Other health strategies include chemoprevention, using either synthetic drugs or naturally occurring agents, that interfere with the multi-step pathway of carcinogenesis]. Chemoprevention can be applied in three different scenarios. Primary chemoprevention entails administering a chemopreventive agent to the general population regardless of individual risk. Secondary chemoprevention involves a more focussed intervention where populations with an inherited or familial risk are targeted and tertiary chemoprevention selects populations following resection of a colorectal cancer, in an attempt to reduce the risk of local recurrence or metastatic spread[1].

Recent randomised clinical trials have shown that Non-steroidal Anti-inflammatory Drugs (NSAIDs) such as the relatively non-selective Cyclooxygenase (COX) inhibitors, piroxicam and sulindac and the relatively selective COX-2 inhibitor, celecoxib, are able to cause regression of adenomas in patients with familial adenomatous polyposis, by up to 100%[2-5]. The administration of aspirin to patients with recurrent colonic adenomas, or previous colorectal cancer, has also been shown to reduce new polyp formation by up to 35%[6,7]. The structures of common NSAIDs can be seen in Fig. 1. Such studies suggest that chemoprevention could become a clinical reality, although to be effective patients would be required to take these drugs for many years. It is well documented that long term administration of NSAIDs such as aspirin is associated with side-effects

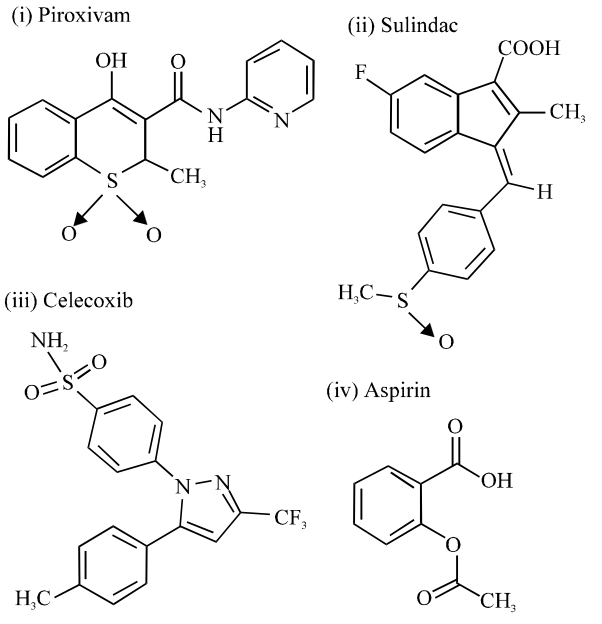

Fig. 1: Structures of common NSAIDs

Corresponding Author: Dr. G. Garcea, Department of Surgery, The Leicester General Hospital, Leicester, LE5 4PW
Tel: 44 116 258 4706 E-mail: gg43@le.ac.uk

such as gastric mucosa inflammation, bleeding, dyscrasias, gastric ulceration and renal impairment. The inevitable morbidity associated with such side-effects may obfuscate any benefit gained from reduction of colorectal cancer risk. The recent worldwide withdrawal of the COX-2 inhibitor, Rofecoxib, further highlights the potential problems of long -term NSAID use. In this short report, evidence is reviewed that supports the notion of polyphenolic phytochemicals being considered an efficacious and safe alternative to NSAIDs.

Why polyphenolic phytochemicals?: Polyphenolic phytochemicals make up a large proportion of the constituents of the human diet, the main sources being fruits, chocolate, vegetables, cereals, legumes and beverages such as tea, coffee and wine[8]. The exact classification and definition of polyphenolic compounds is open to debate and not the focus of this review. This report will centre mainly on three compounds namely curcumin, the major yellow pigment in the spice turmeric, resveratrol, a component of grapes and red wine and tea polyphenols with particular emphasis on epigallocatechin gallate (EGCG) (Fig. 2).

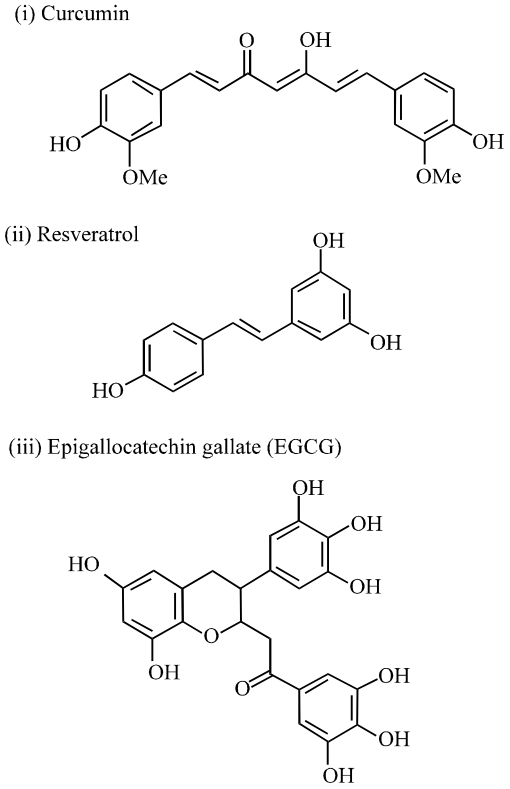

Fig. 2: Structures of a number of common polyphenolic phytochemicals

The conception that polyphenolic phytochemicals possess colorectal cancer chemopreventive properties is based on epidemiological findings that suggest the intake of polyphenol rich foods may delay the onset of cancer via antioxidant activity, induction of phase I and II detoxifying systems or by preventing the formation of carcinogenic precursors, such as heterocyclic amines[9]. More recently there is evidence to suggest that certain polyphenols have a direct inhibitory effect on cancer cell growth by inhibiting proliferation, promoting apoptosis and inhibiting angiogenesis[10,11]. Observational and case-control studies indicate that intake of fruit and vegetables is associated with a lower risk of colorectal cancer[12-18]. The protective effect of high fruit intake is also evident amongst cohorts of individuals who are at high risk of colorectal cancer, including those with previous cancer history, colonic polyps and ulcerative colitis[19]. Several retrospective epidemiological studies have shown that regular ingestion of green tea can be chemopreventive[20-23]. One large Japanese study followed 8552 patients over a nine-year period and observed an average delay of cancer onset of 4 years ($p<0.01$) in individuals who consumed 10 or more cups of green tea daily, when compared to those consuming less than 3 cups daily[24].

Mechanisms of NSAID-and Polyphenolic Phytochemical-Mediated Cancer Chemopreventive Activity: A wide range of polyphenolic agents have been identified and are currently under evaluation using *in vitro* and *in vivo* models of carcinogenesis. To fully understand the potential of these polyphenolic phytochemicals, we must understand how they exert chemopreventive activity. Some of the known mechanisms of NSAID-and polyphenol-mediated chemopreventive activity are outlined in Table 1 and 2, respectively. These data show that both groups of compounds act *via* multiple mechanistic pathways, some of which overlap, for example antioxidation. The exact mechanistic pathways associated with cancer chemopreventive effects are, as yet, unclear. Some of the potential chemopreventive mechanisms associated with curcumin, green tea and resveratrol will now be considered.

Curcumin is a bright yellow pigment derived from the rhizome *Curcuma* Longa (Fig. 2 for chemical structure). It is found in the spice turmeric, which is widely used in Indian cuisine as a colouring and flavouring. Like many polyphenols curcumin has been shown to inhibit COX-2 expression in both human colorectal tumour cell lines *in vitro*[25] and to decrease PGE-2 expression in humans *in vivo*[26]. Curcumin has been shown to inhibit oxidative DNA adduct formation as measured by levels of the

Table 1: Mechanistic targets of NSAIDs potential related to cancer chemoprevention

Therapeutic target	Compound
COX-1 and / or COX 2 inhibition	Piroxicam
	Aspirin
	Sulindac
	Celecoxib (COX-2 only)
Induction of apoptosis	Piroxicam
	Aspirin
	Sulindac
Modulation of LOX	Piroxicam
	Aspirin
	Sulindac
Suppression of prostaglandin synthesis	Piroxicam
	Aspirin
	Sulindac
	Celecoxib
Induction of cell cycle arrest	Celecoxib
	Sulindac
Inhibition of angiogenesis	Aspirin
	Celecoxib

Table 2: Mechanistic targets of polyphenolic phytochemicals potentially related to cancer chemoprevention

Therapeutic target	Compound
COX-2 inhibition	Curcumin
	Black tea
Induction of apoptosis	Curcumin
	Epigallocatechin gallate
Immune system modulation	Curcumin
	Resveratrol
Inhibition of cell signalling pathway via cyclin D1	Resveratrol
Induction of GST Phase II detoxifying enzymes	Resveratrol
	Curcumin
Inhibition of nitric oxide synthase	Black tea extract
	Curcumin
	Resveratrol
Inhibition of oxidative DNA adduct formation	Black tea extract
	Curcumin
Anti-oxidant mechanism	Curcumin
	Resveratrol
	Green tea extract
	Black tea extract

pyrimidopurinone DNA-adduct (M_1G)[27], decrease the expression of the onco-protein beta-catenin[28], induce apoptosis in Colo 320 colon cancer cells and AOM-induced colon tumors[29,30] and induce the glutathione-S-transferase (GST) de-toxification enzyme system[27,31]. Curcumin can also modulate immune system-mediated tumour cell killing by increasing the numbers of intestinal CD4+ T cells and B cells[32].

Resveratrol is a polyphenolic compound found in grapes, peanuts, berries and red wine (Fig. 2 for chemical structure). Experiments using cancer cell lines *in vitro* have shown resveratrol has an anti-proliferative effect[33,34] and in $CaCo_2$ human colon cancer cells, resveratrol induced the accumulation of cells in the S/G2 phase of the cell cycle, reflected by a 70% inhibition of growth[35]. Similarly in HT29 colon adenocarcinoma cells, resveratrol induced cell cycle arrest at the G2 phase *via* inhibition of CDK7 kinase activity[36]. Resveratrol can also preferentially alter the levels of proteins involved in apoptotic pathways. For example, in $CaCo_2$ colon cancer cells, high concentrations of resveratrol activated the proapoptotic protein caspase-3[37]. In HCT116 cells resveratrol has been shown to activate a p53-independent apoptotic pathway that is potentially linked to cell differentiation[38] and to induce both Bax-mediated and Bax-independent mitochondrial apoptosis[39].

Green tea contains a number of polyphenolic phytochemicals known as catechins. Epigallocatechin gallate (Fig. 2), one of the primary catechins, has been shown to induce apoptosis and interfere with cell cycle progression in a number of colorectal tumour cell lines *in vitro*[40-43]. This cytostatic effect was specific to tumour cells alone. Other effects include inhibition of DNA adduct formation[44], preservation of the colonic microflora[45] and electrophile scavenging[46]. Epigallocatechin Gallate (EGCG) has been shown to inhibit DNA topoisomerase I, an enzyme involved in cell survival and DNA metabolism and structure, in numerous human colon carcinoma cell lines[47]. Tea polyphenols have also been shown to induce enzymes involved in Phase II detoxification of dietary carcinogens[48].

With regard to chemoprevention mediated by NSAIDs, it is thought that inhibition of COX-2 activity is an important mechanism of action in colorectal cancer, however, other mechanisms are documented (Table 1) and include modulation of apoptosis, induction of cell-cycle arrest and inhibition of angiogenesis[10,11]. Furthermore, sulindac has been shown to modulate the $-catenin/TCF4 pathway *via* induction of p21 expression[49] and celecoxib can decrease phorbol-ester-induced COX-2 expression and AP-1 DNA binding[50] and can inhibit NF-6B activation[51].

Chemopreventive efficacy of polyphenolic phytochemicals and NSAIDs *in vivo*: The data demonstrate that there is a degree of overlap between the mechanisms of chemopreventive activity of polyphenols and NSAIDs and support the notion that polyphenols are effective chemopreventive agents. But an important consideration is the relative efficacy of polyphenols as chemopreventive agents when compared to NSAIDs *in vivo.*

Studies using genetic models of cancer and carcinogen-induced aberrant crypt foci models suggest that under certain conditions the chemopreventive efficacy of polyphenolic phytochemicals and NSAIDs can be similar (Fig. 3). Indeed, polyphenols such as green tea and soy appear to be more effective than NSAIDs at inhibiting carcinogen-induced ACF *in vivo*. The chemopreventive activity of NSAIDs and polyphenols against carcinogen-induced tumour models *in vivo* are compared in (Fig. 4). These data demonstrate that under

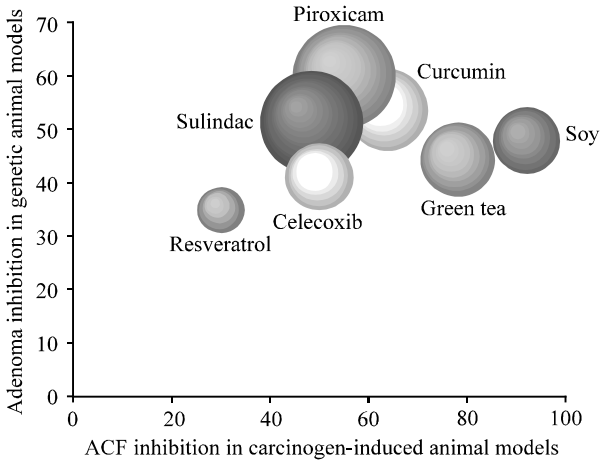

Fig. 3: Comparison of efficacy of NSAIDs and polyphenols in genetic animal models and carcinogen-induced cancer models. Bubble size relates to the number of studies evaluating that particular agent

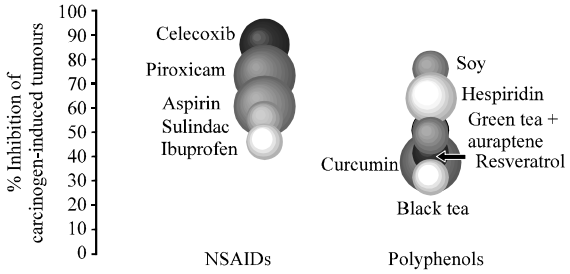

Fig. 4: Comparison of NSAIDs and polyphenols, ranked on potency to inhibit carcinogen-induced tumours. Bubble sizes relate to number of studies evaluating that particular agent

certain conditions NSAIDs such celecoxib can be more effective than polyphenols, although overall both groups of compounds show a similar range of efficacy.

Table 3-5 summarize the current *in vivo* data comparing the chemopreventive effects of curcumin, resveratrol and tea polyphenols to the those of the NSAIDs, piroxicam, sulindac and celecoxib in the $Apc^{Min/+}$ mouse, a model of human familial adenomatous polyposis and the azoxymethane (AOM)-induced adenocarcinoma rat model. Briefly, studies in the $Apc^{Min/+}$ mouse have shown that curcumin and resveratrol can inhibit adenoma formation by up to 70% in the small intestine and 100% in the colon[28,52]. Similar studies using NSAIDs have shown up to 99% inhibition of adenoma formation[53-62]. These data suggest that, depending on the dose and duration of treatment, curcumin and resveratrol are almost as effective as NSAIDs at inhibiting adenoma development. In studies

Table 3: Chemopreventive activity of NSAIDs and polyphenolic phytochemicals in the ApxMin/+ mouse. Treatment efficacy has been determined by inhibition of adenoma development

Compound	Dose (ppm)	Duration (days)	Adenoma development inhibition (%)	Reference
Piroxicam	25-220	7-180	34-95	[53-56, 60]
Sulindac	30-300	7-80	32-99	[53, 57-62, 94]
Celecoxib	150-1500	25-55	27-71	[55]
Curcumin	1000-2000	70-75	6-64	[28, 73, 95]
Resveratrol	100 (in water)	49	70	[52]
Tea extract	1000	70	22	[61]

Table 4: Inhibition of aberrant crypt formation following dietary intervention with NSAIDs and polyphenolic phytochemicals in the AOM-induced colon carcinoma rodent model.

Compound	Dose (ppm)	ACF development inhibition (%)	Reference
Piroxicam	75-400	38-70	[96-99]
Sulindac	100-320	36-53	[98, 100-103]
Celecoxib	1500	41	[101]
Curcumin	2000	42-57	[64, 67, 104]
Resveratrol	200	38	[105]
Tea extract	200-1200	35-57	[63, 66]

Table 5: Decrease of tumour incidence following dietary intervention with NSAIDs and polyphenolic phytochemicals in the AOM-induced colon carcinoma rodent model

Compound	Dose (ppm)	Decrease of tumour incidence (%)	Reference
Piroxicam	200-400	64-85	[106-108]
Sulindac	320	55	[109]
Celecoxib	1500	78-93	[110, 111]
Curcumin	600-40000	25-42	[112-114]
Tea extract	1000	51	[115]

using the AOM-induced adenocarcinoma rat model, curcumin and green tea extracts showed similar efficacy to NSAIDs at inhibiting aberrant crypt foci formation, although NSAIDs were superior at reducing tumour incidence[63-68].

Combined with the apparent lack of unwanted side effects following long term administration, these data suggest that certain polyphenolic phytochemicals may prove a sensible alternative to NSAIDs for use in colorectal cancer chemoprevention.

Bioavailability of polyphenolic phytochemicals: It is known that NSAIDs show a relatively high bioavailability. For example, aspirin is a weak acid that remains largely unionised in the acid environment of the stomach thereby facilitating its absorption[69]. Current preclinical data from *in vivo* models suggests that polyphenols are poorly absorbed and avidly metabolised. Small polyphenols such as caffeic acid are most bioavailable following oral administration showing up to 27% recovery from urine, whereas tea polyphenols are poorly bioavailable showing around 0.00006% recovery from urine[70]. Curcumin is poorly bioavailable and is subject to a rapid first pass metabolism[71] with only trace amounts detectable in the

peripheral circulation following oral administration[72,73]. Similarly other polyphenols such as resveratrol display poor bioavailability[74-76]. A further reduction in polyphenol bioavailability can also occur *via* bacteria-mediated degradation in the large bowel[8,77].

Polyphenols can conjugate with glucuronide moieties and such conjugation may assist in their absorption from the small intestine[78]. In an attempt to increase absorption and consequently bioavailability, polyphenols have been co-administered with compounds such as lipids and emulsifiers[79]. Such a protocol significantly enhanced the absorption of the polyphenol quercetin and might therefore prove beneficial to other polyphenolic agents. The co-administration of curcumin with a pepper constituent has also been shown to increase curcumin absorption by a factor of 20[80].

Certain polyphenols, such as epigallocatechin gallate from green tea, are excreted in bile[81]. Enterohepatic recirculation of bile excreted metabolites, a process that has been postulated following administration of resveratrol to rats[75], might act to sustain therapeutic drug concentrations in the gut following oral administration and may therefore potentiate chemopreventive activity. It should be noted that the major metabolites of polyphenols and their intrinsic chemopreventive efficacy are still largely uncharacterised. It is possible that the beneficial effects of polyphenols are not reliant on their absorption though the gut barrier and that their efficacy may result from a direct anti-oxidative effect on mucosal cells[82], beneficial effects on gastrointestinal micro-flora[83], localised absorption and distribution to gastric epithelial cells, or may be attributable to their metabolites.

The differences in bioavailability of certain NSAIDs and polyphenols do not appear to affect their chemopreventive efficacy in the gastrointestinal tract, for example the bioavailability of curcumin is poor, however, in the $Apc^{Min/+}$ mouse has shown considerable efficacy (Table 3). Although poor absorption of polyphenols is likely to hinder their chemopreventive activity in cells distant from the gastrointestinal tract, such limitations may result in localised accumulation in the gastrointestinal tract thereby dramatically decreasing the risk of untoward side-effects in distant organs.

Clinical studies of polyphenolic phytochemicals: As yet few polyphenolic phytochemicals have been investigated in clinical trials. Curcumin has thus far been the most investigated. Serum levels of curcumin have been shown to be low following oral administration to patient volunteers, with measured levels of <0.03 μM following doses of up to 2 g[80] and 1.75 μM after 8 g oral administration[84]. In one study, no detectable levels of curcumin were found in urine or blood following oral administration to human volunteers at doses from 36-180 mg[72], however, curcumin sulphate was detected in the faeces of one patient at the 180 mg dose level, thus supporting previous work showing polyphenol conjugation can occur in the gastrointestinal tract[85].

Clinical data suggest that curcumin is non-toxic and does not accumulate within the body. Daily oral doses as high as 8 g have been administered to patients for 3 months with no adverse effects[84] and other studies involving curcumin administered at doses of 180 mg to 200 mg daily failed to demonstrate any toxicity[72]. In a recent clinical trial[26] a daily 3.6 g dose of curcumin for 7 days resulted in curcumin accumulation in colorectal tissue to concentrations equivalent to those (5-5 μM) required for pharmacological activity in cells *in vitro*[25,86-90]. This same study[26] also found that patients receiving 3.6 g of curcumin had a significant reduction in tumour levels of oxidative DNA damage ($p<0.05$, student t test) and trace levels of curcumin were only detected in the peripheral circulation one hour after administration of the highest dose. These data show that despite its poor absorption and rapid elimination from the body, pharmacologically active levels of curcumin can be achieved in the colorectal mucosa when administered at high enough doses.

Clinical studies using green tea are relatively limited. The administration of standardised green tea solids (0.6-1.8 g), dissolved in warm water, to human volunteers has been shown to result in a rapid decrease of rectal mucosa PGE_2 levels within 8 h of consumption[91]. Unfortunately, the clinical use of such a regimen may be marred by reports of side-effects including bloating, nausea, vomiting, agitation, dizziness and restlessness[92]. Such side-effects are probably due to the caffeine content within green tea, often up to 7%[92]. Decaffeination is therefore an option, however, caffeine has been reported to enhance the chemopreventive efficacy of green tea[93] and removing it may reduce efficacy.

Recent data suggests that polyphenolic phytochemicals possess colorectal chemopreventive properties in both *in vitro* and *in vivo* models of colorectal carcinogenesis. Indeed, under certain conditions polyphenolic phytochemicals have been shown to be as effective as NSAIDs. The major confounding factor in the development of polyphenols as chemopreventive agents is their poor bioavailability. Although pharmacologically active concentrations are achievable in the intestinal mucosa, the dose required to achieve this may prove unpalatable to patients needing to be maintained on the medication for many years. The poor bioavailability of polyphenolic phytochemicals may,

however, prove to be a great asset. Although, limiting their use solely to the chemoprevention of colorectal cancer, the restriction of polyphenols to the gastrointestinal tract is likely to decrease the risk of untoward side-effects in organs distant to the locus of absorption. It remains to be seen if randomised clinical trials show polyphenols to be less, similarly, or more effective than NSAIDs in human colorectal cancer. The vast number of available polyphenols makes their development into clinical drugs a daunting but exciting project.

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International Journal of Cancer Research, 1 (1): 71-80, 2005
ISSN 1811-9727

Breast Cancer: An Overview

Maria T. Laux
Department of Clinical Sciences, College of Veterinary Medicine,
Cornell University, Ithaca, New York, 14853, USA

Breast cancer is not a new disease. The oldest known reference to it was recorded by the Egyptians. Hippocrates, the father of medicine, described some of its clinical symptoms. At the end of the first century A.D., Leonidas was operating on malignant breast lumps. Circa 130 A.D., Galen developed some clinical criteria for procedures involving the surgery of these tumors. Galen's ideas remained vital until the 16th century.

The early development of epidemiology concentrated on life style. This approach can be traced back at least to 1700, when Bernardino Ramazzini in Italy noticed an unusually high rate of breast cancer in nuns and speculated that it related to their celibacy and childlessness. An 1844 report in Verona followed up some observations containing comparisons of breast and uterine cancer rates from the city's death registry between the years 1760-1839. The analyses suggested that married women were more than twice as likely to die of uterine cancer than from breast cancer, while nuns were 9 times more likely to die of breast cancer than from uterine cancer. The relative risk of breast cancer for nuns was found to be 22 times higher[1].

The Middle Ages and the Renaissance provide an extensive literature relating to breast cancer, including conventional treatments as well as some which were quite bizzare (e.g witchcraft).

In the late 17th century the Frenchman Henri Francois leDran was the first to notice the tendency of breast (and other cancers) to metastasize. LeDran proposed that cancer was a local disease in early stages, but after spreading to the lymphatic nodes signaled a poorer prognosis.

In the late 19th century the first important information was provided that implicated the ovaries as inducers of tumors of the breast. In 1889, the German scientist Albert Schinzer proposed removing the ovaries in the case of breast cancer, in this way attempting to arrest further tumor development.

In Scotland in the early 20th century George Thomas Beatson widely published papers regarding the use of oophorectomy (removing the ovaries) in obtaining positive responses in a few breast cancer patients. The basis for his findings was the observation that removal of the ovaries in cows prolonged lactation. This suggested to him that interference with the ovarian function in patients with breast cancer might also have some favorable effect[1].

Saint Agatha is the patron saint of those who suffer breast disease. She was martyred having her breasts removed in Sicily in the 3rd century[2].

Recent years have seen remarkable progress of clinical research and experimental laboratory results in breast cancer treatment. Scientific investigation has concentrated its efforts to identify and understand the genetic alterations associated with the malignant changes in breast cancer[3].

Breast cancer is common, the leading cause of death in women between the ages of 35 and 54. In North America, breast cancer is the most prevalent cause of cancer deaths among women of all ages. A woman living to age 80 in North America has 1 chance in 9 of developing invasive breast cancer. The number of cases continues to soar[4].

American women are now twice as likely to develop breast cancer than they were a century ago and most of this increase in incidence has occurred over the past thirty years. Since 1973 the incidence of breast cancer among white women has increased by 34% and among black women by 47%[5]. In the US in 1996 alone, 186,000 women learned they had breast cancer and about 46,000 died from it. The log-log plot of incidence versus age shows a straight line increase with every year of life[6].

Breast cancer rates vary considerably among European countries. Highest incidences are found in western and northern European countries and lowest incidences in eastern and southern European countries[7].

Breast cancer incidence has increased steadily in the past three to four decades. Throughout the world this pattern has been observed, while the rate of occurrence varies widely among different countries. Oriental countries like China and Japan have the lowest rates. Because of the lack of uniform occurrence, it is believed that environmental-especially dietary-factors are a major determinant of risk[8].

Japanese, Chinese and other oriental women have a risk to develop breast cancer at a rate of only 20/ 100,000[9]. Oriental women have substantially lower concentrations of estrogens and progesterone and their

height and weight are markedly less than those in other ethnic groups. Height and weight are critical regulators of age of menarche and have substantial effects on plasma concentrations of estrogens. Large increases in the rate of breast cancer occur in populations migrating from nations with a low incidence to those with a high incidence of breast cancer, again indicating the existence of environmental factors[10].

In men breast cancer is about 1/150th as frequent as in women. It usually presents itself as a unilateral lump in the breast and is generally not diagnosed promptly. When male breast cancer is matched to female breast cancer by age and stage, its overall prognosis is identical. Approximately 90% of male breast cancers have estrogen receptors and approximately 60% of cases with metastatic disease respond to endocrine therapy[11].

The three dates in a woman's life that have a major impact on breast cancer incidence are:

- age of first menstruation (menarche)
- age at first full-term pregnancy
- age of menopause[12]

Women who experience menarche at age 16 have only 50 to 60% of the lifetime breast cancer risk of women who experience menarche at age 12. Similarly, menopause occurring 10 years before the median age (52 years), whether natural or surgically induced, reduces lifetime breast cancer risk by about 35%[13]. Women who have a first full-term pregnancy by age 18 have only 30 to 40% of the breast cancer risk faced by others. Thus, length of menstrual life-particularly the fraction occurring before the first full-term pregnancy-is a substantial component of the total risk of breast cancer[12].

The role of diet in breast cancer etiology is controversial. While there are associative links between total caloric intake and breast cancer risk, the strongest link is with high dietary fat intake[8]. However, within the range of dietary fat intake common in Western cultures, there is no convincing evidence that variations in dietary fat alter breast cancer risk[14].

There is a risk associated with even moderate alcohol intake; the mechanism is unknown. Recommendations favoring abstinence from alcohol must be weighed against social pressures and the possible cardioprotective effects of moderate alcohol intake[15].

Women without functioning ovaries who never receive estrogen replacement therapy do not develop breast cancer[16]. The potential role of exogenous hormones in breast cancer is of extraordinary importance because millions of American women regularly use oral contraceptives. The most credible analyses of oral contraceptive use suggest that these agents cause little, if any, increased risk of breast cancer. The data suggest that the use of oral contraceptives is highly protective against malignancy in general, but has little impact on breast cancer[17].

Far more controversial are the data surrounding Hormone Replacement Therapy (HRT). HRT with estrogens alone, usually in the form of equine conjugated estrogens, provides less than the physiologic equivalent of premenopausal estrogens but is associated with an increased risk of endometrial cancer, a reduction in the symptoms of estrogen deprivation and a reduction in deaths due to cardiovascular disease. Analyses suggest a small increase in breast cancer incidence associated with HRT, particularly with high dosage and a long duration of treatment. For the average woman, the negative effect on the breast is far outweighed by the positive effects on the bones and heart. Preliminary data suggest that there is a reduction in the risk of colon cancer as well[18].

The addition of progestin to HRT regimens drastically reduces the risk of endometrial cancer[19]. It is not clear whether the protective effects against cardiovascular and osteoporotic diseases are altered. However, progestins are copromoters of breast cancer in model systems and an increased risk of breast cancer is possible. Whether a history of previous biopsy findings of atypical hyperplasia or *in situ* carcinoma or a strong family history of breast cancer alter the risk-to-benefit ratios for HRT is unknown. It is likely that the average woman benefits from HRT[20,21]. HRT is undergoing paradigm shift. Recently doctors have increasingly begun to recognize harmful effects in treatments once thought to be beneficial to menopausal women. A new study done by Christopher Li adds more weight to the link between hormone use and breast cancer. The study found that two types of cancer which increased with hormones are lobular and lobular-ductal mixed. Both types of invasive cancer have increased 65% from 1987-99[22]. Ironically, JAMA published a goverment study purporting that combined estrogen-progestin treatment improves mental outlook and memory.

Hormone replacement therapy increases breast density. Dense tissue contains more glandular tissue and is a risk factor for breast cancer. The explanation might be that the excess of glandular tissue in the dense breast provides much more cells potentially able to transform into neoplastic cells. High density of breast can lead to impaired sensitivity of mammographic screaning[23].

Two studies of twins showed that the population variation in the percentage of densew tissue revealed during mammography has high heritability at any given age[24].

In addition to the other factors, radiation may be a risk factor in younger women. Women who have been exposed to radiation before age 30 have a substantially increased risk of breast cancer, whereas radiation exposure after age 30 appears to have minimal effect on the breast[25].

Two complementary approaches have been taken to elucidate at a molecular level the process of malignant progression of human breast cancer. One approach has focused on the genetics of somatic mutations frequently found in primary breast tumors[25]. The other approach has focused on the expression of specific gene products present in primary breast tumors or breast cancer cell lines[26].

The activation of expression of certain growth factors, growth factor receptors and nuclear proteins occurs as a direct consequence of gene amplification of cancer cells. These *loci* include the c-myc protooncogene on chromosome 8q24[27], an amplicon on chromosome 11q13 that contains the INT2 (FGF3), HST1 (FGF4)[28] PRAD1 and EMS1 genes[29] and the c-erb B2 protooncogene on chromosome 17q[30]. In addition, BEK (bacterial expressed kinase) and FLG (FMS like gene), two members of the FGF receptor (FGFR) gene family, are amplified in 11.5% and 12.7% of breast cancer, respectively[31].

By far the most frequent type of mutation found in primary breast cancers is Loss of Heterozygosity (LOH). This is a common feature of many kinds of malignancy and occurs as a consequence of either interstitial deletions, chromosome loss or aberrant mitotic recombinational events. It is thought that LOH reveals within the affected region of the genome the presence of a recessive mutation in the remaining allele of a tumor supressor gene or genes[32]. At present, 20 of the 41 chromosome arms in the human genome have been shown to be affected by LOH in primary breast tumors. However, only two of the putative genes for LOH have been identified, p53 on chromosome 17p12[33] and Rb on chromosome 13q14[34].

Tumor suppressor genes are believed to be involved in the normal suppression of cellular proliferation[32]. Studies have focused on defining the regions of each chromosome arm containing putative target tumor suppressor genes and will lead to the recognition of their function and, eventually, whether or not they are affected by LOH[35].

Familial breast cancer occurs in about 5% of patients. Analysis of families with a history of breast cancer demonstrates the existence of a breast cancer susceptibility gene on the long arm of human chromosome 17. This gene was named BRCA1. Mutations of the BRCA1 gene are responsible for 45% of families with a high incidence of breast cancer and at least 80% of families with an increased incidence of both breast and ovarian cancer[36].

The BRCA1 gene has been cloned and found to be a large gene that shows only limited homology to other known genes[37]. Near the amino terminus of the predicted protein is a RING-finger motif associated with DNA binding proteins and gene regulation. Virtually all inherited mutations cause the BRCA1 protein to be prematurely truncated, in keeping with its predicted role as a tumor suppressor gene[38].

DNA sequencing has been used to identify mutations within the BRCA1 gene. Mutations were identified in 16% of women with a family history of breast cancer. The rates were found to be higher among women from families with a history of both breast cancer and ovarian cancer[39]. There is some evidence to suggest that mutations at the 5' end of the gene carry a higher risk of ovarian cancer than those at the 3' end. BRCA1 is infrequently mutated in sporadic breast or ovarian cancer, although LOH in regions adjacent to BRCA1 is common, suggesting that other genes close to BRCA1 may be important in sporadic forms of both types of tumor[40].

Another breast cancer susceptibility gene, BRCA2, on chromosome 13q12-13 has been recently cloned and germline mutations in breast cancer families have been identified[41]. Mutations in BRCA2 appear to account for an additional 45% of familial breast cancer. BRCA2 is associated with a lower risk of ovarian cancer than BRCA1 and a higher risk of male breast cancer[11]. Somatic mutations of BRCA2 in sporadic breast and ovarian cancer are very rare. Between them, BRCA1 and BRCA2 may be responsible f or about 90% of hereditary breast cancers[40].

In patients with breast cancer, amplification of the HER-2/neu gene has proven to be a significant predictor of both overall survival and susceptibility to relapse. The human homologue of the rat neu gene, called c-erbB2 or HER-2, shows extensive homology to the epidermal growth factor receptor c-erbB1[42]. Amplification results in overexpression of gene product. The product of the c-erbB2 gene together with the products of related genes c-erbB3 and c-erbB4, were shown to act as receptors for the ligand heregulin. It is likely that heregulin-mediated activation of these receptors plays a role in breast cancer. Also, increased production of the normal protein products of amplified proto-oncogenes contributes to the development of the malignancy[43].

Defects in other genes, such as p53, the ataxia telangiectasia gene and other as yet unidentified breast cancer susceptibility genes are likely to explain the predisposition to breast cancer in other familial cases.

Alterations in at least five separate genes can result in an increased risk of developing breast cancer. As an example is the rare presentation of breast cancer called Li-Fraumeni Syndrome (LFS). This syndrome occurs when three first degree relatives develop cancer under the age of 45 and one of the three cancers is a sarcoma. In Li-Fraumeni Syndrome, the most frequent malignancy is breast cancer[44]. Affected members of LFS families frequently carry germline p53 mutations[45].

The most commonly mutated gene identified in human breast cancer is p53[46]. The role of this gene is to produce phosphoprotein p53 in the nucleus. Loss of heterozygosity or overexpression of mutated p53 protein occurs in about 30-55% of human breast cancers[47]. Mutations of p53 render the cell unable to die of apoptosis. Alterations of p53 showed a poor response to endocrine therapy. Both endocrine and many chemotherapeutic agents act by inducing apoptosis. Consequently, the turning off of p53 makes induction of apoptosis impossible, resulting in a poor response to chemotherapy[48].

In the Retinoblastoma (RB) gene, loss of heterozygosity occurs in about 25% of primary breast cancer patients and 10-20% of them show evidence of gene inactivation[49]. There is no data available on the relation between loss of heterozygosity of the RB gene and response to treatment[49].

The myc gene may spontaneously stimulate proliferation. C-myc, with amplification, is known to bind and inactivate the Rb-1 gene product and, in this way, to decrease tumor suppression[50].

Expression of nm23, a putative metastasis suppressor gene mapped to chromosome 17q21, has been detected in human breast cancers[51]. Already studies have demonstrated a significant association between reduced nm23 expression and aggressive cancer behavior[51].

Mutations in the ras gene, which frequently are found in other cancers, are not found in breast cancer, although increased levels of Ha-ras have been observed[76].

Growth factors, differentiation factors and hormones are important elements in the proper development and functioning of multicellular organisms. Polypeptides such as these work as intercellular messengers governing proliferation, differentiation and the process of metabolism. Growth factors have the ability to bind to factor-specific cell-surface receptors exhibiting protein tyrosine kinase activity[52].

Receptor Tyrosine Kinases (RTKs) all have similar molecular structure. All RTKs are dimers and contain a glycosylated extracellular ligand binding domain, a hydrophobic transmembrane region and a cytoplasmic domain containing a tyrosine kinase catalytic domain[52].

RTKs have been classified into nine subgroups based on sequence similarity and distinct structural characteristics. These include the EGF receptor family, the insulin receptor family, the PDGF/MCSF-1/Steel receptor family, the VEGF family, the hepatocyte growth factor family, the neurotrophin receptor family, the FGF receptor family, the Eph-like receptor tyrosine kinase family and the Axl-receptor tyrosine kinases[53].

RTKs are all activated in the same manner: ligand binding to the extracellular domain causes receptor dimerization and oligomerization. Oligomerization of receptors will increase the activity of tyrosine kinases, which will lead to enhancement of ligand binding affinity. Oligomerization leads to the autophosphorylation of the receptor as well as of the cellular substrates. Upon kinase activation, RTKs are able to turn on a number of different intercellular signaling pathways that will ultimately lead to cell proliferation, differentiation and metabolic activity.

The importance of RTK signaling in the determination of cellular activity and cell fate has implications for carcinogenesis. Within receptor tyrosine kinases a number of mutations can occur which can lead to altered signaling. RTKs can be constitutively activated through mutations in the binding domain that cause a conformational change leading to the constant oligomerization/activation of receptors. Deletions or mutations within the cytoplasmic domain will lead to increased receptor signaling through increased tyrosine kinase activity or enhanced substrate affinity. Nevertheless, most mutations resulting in a constitutively activated receptor tyrosine kinase are not directly transforming.

In cancer, involvement of RTKs has been linked to their autocrine activation and receptor overexpression. This combination results in unregulated receptor signaling, increased signaling of proliferation and the overcoming of apoptosis[52,53].

Epidermal Growth Factors (EGFs) as well as Transforming Growth Factor Alpha (TGF"), both of which can activate EGF receptors, are produced locally in normal and malignant tissues. Malignant tumors frequently contain higher numbers of EGFR than normal tissues, but there is no agreement on the prognostic value of EGFR[54]. Some studies suggest that expression of EGFR is associated with a lack of response to endocrine therapy in recurrent breast cancer[55]. Acquired tamoxifen

resistence is associated with an increase in EGFR numbers accompanied by loss of Estrogen Receptors (ER) and Progesterone Receptors (PGR)[56].

Insulin-like growth factors IGF1 and IGF2 are potent mitogens for breast cancer cells[57]. Apart from estrogens, they are the most powerful growth stimulators *in vitro*. The growth effects of both are mediated via IGF1 receptors, which have been demonstrated to be present in 67-93% of human breast cancers[58]. So far, no data are available on the relation between tumor IGF1R levels and response to therapy in patients with breast cancer. With respect to IGF2, it was suggested that its overexpression may be capable of mediating malignant progression in human breast cancer. The blockade by monoclonal antibodies of IGF1R and downregulation of IGF2 by tamoxifen or estrogens results in growth inhibition, suggesting that IGF2 expression is correlated to breast cancer growth and both IGF2 and IGF1 may be involved in hormone independence[59].

Breast cancer is termed a hormonally driven tumor. This indicates that the development and growth of the tumor is spurred by the body's hormones. The regression of an advanced mammary tumor following surgical castration of ovaries was observed as early as the end of nineteenth century. This fact indicated a dependance of tumor upon the presence of female sex hormones[60]. Such observations provided a base for the introduction of hormonal therapy in treatment of mammary cancer. The practice of castration was abolished with the development of a pharmacological alternatives introduced various anti-estrogenic drugs, the most popular of which is tamoxifen. In breast cancer it is the hormone estrogen that primarily drives its development and growth. On balance, estrogen is an enormously healthful hormone. Estrogen, beside its effects on the secondary sex characteristics in women, also has a variety of roles that promote health: it protects the heart, protects against bone loss by helping to absorb calcium from bloodstream, stimulates water retention and body oil lubrication, so the skin remains youthful. Recently, scientists proved that estrogen interacts with nerve growth factors to protect brain cells from degenerating.

When Elwood Jensen discovered the estrogen receptor-ER-" in 1958, for many years thereafter it was known as the classical receptor.

Jensen's explanation of the two steps mechanism of steroids hormones was confirmed by others' studies. This mechanism involves the activation of receptor proteins by the hormone estrogen and their subsequent binding to hormone response elements in target genes, thus regulating their expression.

In the late 1970's Jensen's discoveries led to the standard clinical procedure of analyzing the receptors on the breast tumor tissue to determine therapy choices. It was known that some breast cancers are hormonally driven, but only in approximately one-third of them does the depravation of estrogen lead to remission. Jensen's study proved that only patients whose cancers have substantial receptor levels benefit from treatment that reduces the amount of estrogen in the body.

The dogma that only one such receptor ER-alfa, existed was finally abolished by a Swedish group in 1996 by discovering receptor ER-$. Their studies proved the existence of two independant ER genes in the human[61,62]. This finding forced scientists to search for possible new treatment,some perhaps utilizing ER-" and ER-$-specific ligands.

Breast cells have receptors on their surface, each one capable of receiving a single message. Once the message is received, it is transmitted to the center-nucleus, where it is processed and then directed to a specific gene. However, sometimes cancer cells produce receptors that are abnormal and behave as if they've been stimulated by growth factors though none are present.

The steroid hormones (testosterone, estrone, progesterone, cortisol and aldosterone) all act on target cells by binding with them. They have a high affinity to a cytoplasmics receptors protein which forms with them a hormone-receptor complex. Consequently, a change in conformation of the receptor occurs, resulting in an activated complex which triggers an enhancement of genetic description. This initiates a spectrum of processes responsible for manifestation of hormonal responses.

When estrogen attaches to receptor, it directs the cell to carry out a task, such as ovulation, menstruation, pregnancy, or lactation. Estrogen's activity was one of the first examples of the critical process called cell signaling, the means by which all cells in the body receive orders, including orders to grow and divide.

Some, but not all, breast cancers cells carry estrogen receptors. When a breast cancer cells carries a receptor, the grow and divide message from an estrogen molecule can be crucial for its proliferation.

Estrogen Receptors (ER) status was the first of the biological markers to be studied.

About 40% of breast cancers in premenopausal women and 60% in postmenopausal women are ER positive. The original assay methods were biochemical, using a radioligand assay, but have now been replaced immunochemical assays using a monoclonal antibody raised against an ER-related protein[63].

It is recommended that all breast cancer patients have their ER status estimated and recorded. Tumors that are ER positive tend to be less life threatening than those that are ER negative, precisely because they respond to estrogen therapy. Natural products, such such as isoflavones from soy, which block estrogen receptors may emerge as obvious candidates for drug development.

Progesterone, the hormone that becomes active in the second half of the menstrual cycle, inhibits cell division in the uterus and breast and so is thought to counteract the stimulation of cell growth caused by estrogen.

Progesterone receptors depend upon an intact ER pathway in order to be expressed. Therefore their status tends to correlate with ER status. Patients with estrogen-positive tumors have between a 50 and 70% chance of responding to hormonal therapy and this increases to over 70% in patients whose tumors have both estrogen and progesterone receptors[63].

The major source of estrogen in premenopausal women is the ovaries. Levels of estrogen in postmenopausal women are much lower and their estrogen is synthesized peripherally from androstenodione which is produced in adrenal gland. Such estrogen is found principally in fat (including breast fat), but also in the skin, muscle and liver. The production of estrogen requires the presence of hormone aromatase.

Tamoxifen is a synthetic hormone which acts primarily by binding to the estrogen receptors. It is the most widely used hormonal treatment for breast cancer.

Studies done by Clark *et al*. showed the relationship between estrogen receptors, progesterone receptors and a variety of patient characteristics in 2,977 women with primary breast cancer[64]. Older women are were likely to be estrogen-receptor positive than younger women. When patient age and menopausal status were analyzed together age was found to be primary determinant of increased estrogen receptors concentrations. There appeared to be no correlation of progesterone receptors concentration with either age or menopausal status when these variables were analyzed separately. However, premenopausal women had higher progesterone concentrations than postmenopausal women when patients of the same age were compared, perhaps reflecting greater estrogen-mediated synthesis of progesterone receptors.

Further understanding of the action of hormones which positively affect the breast and consequently reduce the risk of malignant development will be the task of future research. The evidence shows that estrogen is the hormone most connected to breast cancer, enhancing proliferation more often and more rapidly than others. Thus, if a mutation, inherited or triggered by a carcinogen, lies embedded in the DNA, cancer cells are more likely to proliferate when high estrogen levels are present.

Perhaps blocking the action of estrogen by partially replacing it with less aggressive hormones such as phytoestrogenes derived from plants can slow down or stop the harmful effects of estrogenes exposure throughout life's cycles.

Recent studies have concentrated on the superfamily of growth factor receptor tyrosine kinases in order to understand the biochemistry of of human cancer. Protein kinases transfer phosphates from adenosine triphosphate (ATP) to other proteins.

The Epidermal Growth Factor (EGF) subfamily comprises the focus of research in laboratories worldwide. It includes the EGF receptor, the Neu tyrosine kinases (also known as Erb2 or HER2) the Erb3 (or HER3) protein and Erb4 (or HER4) protein.

The EGF receptor is highly overexpressed in some human tumors. Breast cancer cell lines express differing amounts of EGF receptors tyrosine kinases and Neu tyrosine kinases.

Epidermal Growth Factor Receptors (EGFR) is a cell membrane protein which is related to an oncogene product, v-erbB, originally named after the erythroblastic virus which causes cancer in chickens. Binding of its ligand, EGF, promotes the growth of cancer cells, including breast cancer cells, grown in culture.

ErbB2 or HER2 protein is a cell membrane receptor and is the product of the neu oncogene. It was discovered that this oncogene had not mutated like most others. Usually a mutation causes a gene to produce the normal protein, but in abnormally high amounts. This phenomenon is known in molecular biology as overexpression.

When the Neu gene is mutated and the protein overexpressed, the cell is overloaded with signals that cause it to grow out of control-to become cancerous. A typical breast cell carries about 50,000 HER2/neu receptors on the surface. When the gene is mutated, the number jumps to between 1 and 1, 5 million[65].

Just as certain breast and ovarian,cancer cells overexpress HER2/neu, cells in several types of cancer overexpress the EGF receptors.

The status of progesterone receptors always correlates with status of estrogen receptors with a given cells.

Steroid hormones are an important category of hormones involved in the growth regulation of breast cancer. Estradiol, a natural steroid, is one of the most powerful tumor growth stimulating factors. Still the mechanism of hormonal dependence in breast cancer is not completely understood[66].

Breast cancer patients having high levels of Estrogen Receptors (ER) and Progesterone Receptors (PR) receive more benefit from endocrine adjuvent therapy than patients with ER/PR negative breast cancer[67].

The rationale for endocrine therapy has been the reduction of estrogenic stimulation of cancer growth. Tamoxifen has been the principal agent used to block binding of estrogens to their receptors. In advanced breast cancer, activity of tamoxifen can be enhanced by use of prednisone[68].

The relative role of viruses in causing human breast cancer remains ambiguous, but recent studies have established some of their involvement. The natural history of spontaniously occuring cancer suggests a multistep process of cellular evolution repeated selection of rare, sensitive cells with some potential for growth advantage. Some observations suggest that a tumor virus acts as a cofactor in the multiple cellular oncogenes involved in the evolution of cancer. Viral cellular transformation is a heritable, stable change in the control of cell proliferation and differentiation.

The discovery that a particular virus MMTV (Mouse Mammary Tumor Virus), in a certain strain of mice was able to cause mammary tumors led to the question whether similar agent may play a role in human breast cancer[69]. Pogo's findings led to further investigation of viral particles of MMTV-like LTR seguences in human breast cancer and normal breast tissue. Tissue studied by Pogos group originated from highly invasive ductal carcinomas discarded surgically from tumors and from normal tissue during reductive mammoplasties, as a comparison. Detection of MMTV-like LTRs was positive in 41.5% of human breast cancers and in none of the normal tissue analysed[70].

The metastic process is inherently inefficient. Like many aspects of cancer, metastasis is a complicated and poorly understood process. The inefficiency of the metastatic process leads naturally to the question of whether metastasis is a random or a specific process. Clarke's study found that only a minority of breast cancer cells had the ability to form new tumors. After isolation from patients tumorigenic cells CD44 CD24 were identified. It was found that a few as 100 cells of this phenotype were able to form new tumors in mice. Tens of thousands of cells with alternate phenotypes failed to produce new tumors. Clarke determined that only one to fifteen% of breast cancer cells were capable of forming new malignant tumors[71].

Future studies will facilitate the elucidation of pathways that regulate the cells which determine tumor development and survival. Effective therapies could be based on such studies to target and eliminate all tumorigenic (tumor initiating) breast cancer cells.

The skeleton is the most common distant site to which breast cancer spreads (most frequently in the thoracic and lumbar spine). With advanced breast cancer, metastases to the bone can approximate 70% of cases[72].

Inoperable tumors are initially treated with systemic therapy-chemotherapy for premenopausal women and hormone (endocrine) therapy for postmenopausal women. Tamoxifen is the main agent for endocrine therapy[56]. Treatments for advanced breast cancer are not yet curative.

The majority of papers do not show an association between steroid receptor status and response to chemotherapy[73]. Improvements in cytotoxic chemotherapy concentrate on reduction of toxicity and the search for new, strong, selectively working bioactive agents.

Breast cancer is a complex disease with many molecular roads. A new way to find cancer genes is to know the signal transduction pathways to which they belong. Different pathways mean different proteins. Proteins implicated in breast cancer include receptors, growth factors, enzymes, cell cycle regulators and adhesion molecules. Perhaps proteomics will be the key in solving the cancer enigma.

ACKNOWLEDGEMENTS

I wish to thank Paulina Klimkiewicz for her excellent secretarial work. I apologize to those investigators whose contributions were not cited because of space limitations.

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