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TABLE OF CONTENTS

Volume 1 Number 2 June 2010

Research

Review

Case Report

Journal of Cancer Therapy (JCT)

Journal Information

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Improved Detection of Cervical Cancer and High Grade Neoplastic Lesions by a Combination of Conventional Cytology and DNA Automated Image Cytometer

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ABSTRACT

***OBJECTIVE*: *To reduce false-negative rates of population based cervical screening programs employing conventional cytology in combination with automated DNA image cytometer.* ***METHODS*: *Involved cervical samples from a total of* 3603 *women were taken by a cervix brush and then placed into a fixative solution. The cells were separated from mucus by mechanical and chemical treatment after which they were deposited onto microscope slides by a cytospin. Two slides were prepared from each case; one slide was stained by Papanicolaou stain for conventional cytology examination, while the other slide was stained by a DNA specific and stoichiometric stain. The latter slide was used to determine the relative amount of DNA in the cell nuclei in order to assess the ploidy status of the epithelial cells. Enrolled in the study,* 157 *women were followed by colposcopy examination where punch biopsies were taken from the visible lesions or from suspicious areas. The results of the conventional cytology were then compared to the DNA image cytometer for all samples.* ***RESULTS*: *Histopathology diagnosed* 51 *lesions from the* 132 *biopsied cases as CIN*2 *or higher, including* 27 *CIN*2, 16 *CIN*3 *and* 8 *invasive cancers. Conventional cytology correctly identified* 29 *of the* 51 *high grade CIN and invasive cancer, while DNA image cytometer correctly identified* 38 *high grade CIN and invasive cancer using the criterion that at least three cells were found on the slide that contained DNA amount in excess of* 5c. 42 *out of* 51 *high grade CIN and invasive cancer were found by conventional cytology in combination with DNA image cytometer. Sensitivities were* 56.8%, 74.5% *and* 82.4%, *while specificities were* 86.2%, 81.5% *and* 81.5% *in conventional cytology, DNA image cytometer and combination both cytology and DNA image cytometer respectively.* ***CONCLUSION*: *The study demonstrated that screening for high grade neoplastic lesions and cervical cancer by DNA image cytometer or combination of conventional cytology and DNA image cytometer is more sensitive than conventional screening approach.*

Keywords*: *Cervical Intraepithelial Neoplasia* (*CIN*), *Conventional Cytology, Image Cytometry, Ploidy, Aneuploid Cells, Invasive Cervical Cancer

1. Introduction

The cervico-vaginal cytologic test is a powerful tool for detecting cancerous and precancerous (neoplastic) cervical lesions. In countries where well organized population based screening programs were implemented, the incidence of invasive cervical cancers and mortality due to cervical cancer have dropped dramatically [1-3]. However, there are many countries in the world that population based screening has not yet been performed due to the relative high cost and lack of skilled technologists.

The correct diagnosis of high grade cervical intraepithelial neoplasia and invasive cancer is a difficult task in the cytological evaluation of cervico-vaginal smears [4-6]. The performance of several laboratories was summarized by van der Graaf *et al.* [7] who shown that only about half of biopsy-documented invasive cancers were appropriately recognized by conventional cytology of cervical smears. To increase the diagnostic accuracy and to avoid mistakes by cytopathology, several cytometry based systems have been developed over the past three decades for the detection of abnormal DNA content of the cervical epithelial cells [8-11].

The study related to the investigation to determine

conventional cytology in combination with ploidy status assessment of epithelial cervical cells by a fully automated image cytometer could be used for an effective detection of high grade of cervical intraepithelial neoplasia and invasive cervical cancer.

2. Materials and Methods

2.1 Sample Collection and Sample Preparation

The study involved a total of 3,603 women from the Yiling region (Hubei Province, China) and included 1/3 women undergoing their routine screening, as well as 2/3 women undergoing their first screening test. The women were invited to come to either Yiling Women and Children Hospital or to one of the several local hospitals. The samples were obtained by employing a cervix brush. After sample was taken, the brush tip was immediately immersed in 20 ml of fixative (Landing Medical High-Tech Company, Wuhan, China**)** in 30 ml plastic vials. All vials from the collection sites were further processed by first adding Dithiothreitol (DTT, Sigma) to the final concentration of 0.1% of DTT. The cell suspensions were then mildly agitated for 1 hour to release cells from the brush into the suspension and to disaggregate the cells from mucus and cell clusters. The cells were then washed twice with 50% alcohol. Two slides were then prepared from each specimen by cytospinning the cells onto microscope slides forming a uniform monolayer. The cytospin preparation contained on average 10,000 cervical cells deposited in the middle of the slide. One of the two slides was stained by the Papanicolaou stain for conventional cytology reading of the slide, and the other slide was stained with the DNA specific and stoichiometric (Feulgen-Thionin) stain (12) for the ploidy status assessment of the cells on the slide by image cytometry method.

Women presenting any cytological abnormalities were directed to repeated cytology and for colposcopy examination. Of over 157 invited women, 132 women came for colposcopy examination and punch biopsies were taken from the visible lesions or from suspicious areas.

2.2 Cytology

All Papanicolaou stained slides were examined by two cytopathologists. The smears were classified into one of the five groups according to the Bethesda system: 1) within normal limits; 2) with ASCUS (atypical squamous cells of undermined significance); 3) low-grade squamous intraepithelial lesion (LSIL); 4) high-grade squamous intraepithelial lesion (HSIL); and 5) squamous cell carcinoma.

2.3 Image Cytometer

All Thionin-Feulgen stained slides were scanned by the Lanidng DNA image cytometer. The images of the cell nuclei were projected onto the CCD that was positioned in the primary image plane of the 20 times objective, resulting in an effective pixel size of 0.34 um × 0.34 um (~ 0.1 um^2). A typical image of the nucleus of a cervical epithelial cell is represented with 700-900 picture elements (pixels). The image of each cell nucleus was captured in the exact focus and the nuclear material was segmented from the background. For each nucleus, 99 nuclear features were calculated including morphological features, photometric features, discrete texture features, Markovian and non-Markovian texture features, run-length features and fractal features. These features were then used to identify objects as true cells or "junk" (overlapping cell nuclei, out of focus cell nuclei, cellular debris) as well as to classify the nuclei to belong to different cell types. The system was trained to perform this task in a fully automated way. The mean integrated optical density (IOD) value of the 2c diploid cells from each slide was used to normalize the optical density features to eliminate any stain intensity variations within the slides.

On average, from each slide approximately 5,000 quality images of cell nuclei were collected and stored in the computer memory of the cytometer. The nuclei, determined by the system to belong to the epithelial cells, were used to calculate and plot the DNA distribution histograms. The histograms were called normal if corresponded to diploid cells with a low proliferation fraction ($S + G_2 + M$) according to the classification of Auer et al. [13]. All other histograms suggesting the presence of: 1) any cells with DNA > 5 c; or 2) diploid cells with a very high proliferation rate where 10% or more of the total cells were found in the proliferation fraction; or 3) a population of aneuploid stem cells, were called potentially abnormal and the women corresponding to any of abnormal histogram were called for a colposcopy examination. All women, correlated with normal histograms were not further examined unless any atypia was found by conventional cytology.

2.4 Pathology

132 biopsy specimens were taken from suspicious areas for histo-pathological diagnosis. Pathology report of each specimen was generated independently by two experienced pathologists.

2.5 Statistical Analysis

Sensitivity and specificity are calculated using the following formula:

$$\text{Sensitivity} = \frac{\text{True Positive}}{\text{True Positive} + \text{False Positive}} \times 100\%$$

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative} + \text{False Negative}} \times 100\%$$

3. Results

3.1 General Results

Of the total of 3.603 smears, conventional cytology found 3,368 smears were within normal limit (93.5%), and 233 had some form of cytological atypia (6.5%) including 180 with ASCUS (5%), 36 with LSIL (1%), 19 with HSIL (0.5%). Of these only 157 women were available for follow up by histopathology. Of the 3,206 cases 3,603 (91.1%) DNA histograms were considered normal and 397 (8.9%) cases were judged suspicious using the above criteria. Of these, 329 had 1-2 cells with DNA amount greater than 5 c and the rest were found with 3 or more cells with DNA amount greater than 5 c or aneuploid stem cells (**Table 1**).

3.2 Comparison of the Results of Conventional Cytopathology with DNA Image Cytometer

Table 2 shows the results of the 132 women that were examined by colposcopy and had taken punch biopsies. Pathology diagnosis by two histopathlogists was used as the 'truth' for sensitivity and specificity (false negative and false positive rate) calculations. There were 51 cases of CIN2/CIN3/CIS/Ca lesions and 81 cases of Normal/benign/CIN1 in total of 132 cases. There were 8 cases of invasive cancers, 16 cases of CIN3/CIS and 27 cases of CIN2 lesions diagnosed by histopathlogists for the total of 51 lesions of CIN2/CIN3/CIS/Ca type. If conventional cytology were to be used to refer all cases of LSIL/HSIL/Ca to colposcope and biopsy, 29 cases (including 4 LSIL without any > 5 c aneuploid cells) were corrected identified and 22 from the total 51 cases were missed.

For DNA image cytometer, using the criterion that 3 or more cells with the DNA amount exceeding 5 c is sufficient to call the sample suspicious to be examined by cytologist, this approach correctly identified 38 of 51 CIN2/CIN3/CIS/Ca lesions. Conventional cytolgy in combination with DNA image cytometer, 42 of 51 CIN2/CIN3/CIS/Ca lesions were correctly identified using the criterion that 3 or more cells with the DNA amount exceeding 5 c or LSIL or HSIL without 3 or more > 5 c cells.

From the data presented in **Table 2**, sensitivity and specificity of conventional cytology, DNA image cytometer and conventional cytology combined with DNA image cytometer were calculated for high grade lesions of CIN2, CIN3/CIS and invasive cancer. Sensitivity of conventional cytology was 56.8% at 86.4% specificity. The criterion of at least 3 cells containing DNA amount greater than 5c would give sensitivity and specificity values of 74.5% and 81.5%, respectively, while for the cytology combined DNA image cytometer were 82.4% and 81.5%, respectively.

Table 1. The results of TBS diagnosis and DNA ploidy analysis in 3603 cervical samples

DNA ploidy Analysis	Cytology				
	Normal	ASCUS	LSIL	HSIL	Total
Normal	3112	90	4	0	3206
1-2 > 5c aneuploid	244	75	8	2	329
≥3 > 5c aneuploid	12	15	24	17	68
Total	3368	180	36	19	3603

Table 2. Comparative results of histopathology, conventional cytology and DNA image cytometer of 132 biopsy cases

Pathology	Conventional Cytology				DNA Image Cytometer Positive (aneuploidy > 5c)		
	Normal	ASCUS	LSIL	HSIL	Negative	1-2 cells	3 or more cells
Norma/benign (47)	2	42	3	0	2	39	6
CIN1 (34)	3	23	5	3	2	23	9
CIN2 (27)	4	10	11	2	1	9	17
CIN3 or CIS (16)	0	5	6	5	1	2	13
Invasive cancer (8)	0	3	3	2	0	0	8

4. Discussion

Similarly to other studies [14,15], DNA image cytometer could increase sensitive in detecting high grade CIN lesions and cervical cancer. In this study, histopathology diagnosed 51 lesions from the 132 biopsied cases as CIN2/CIN3 or higher, of which there were 8 invasive cancers. Conventional cytology correctly identified 5 of the 8 invasive cancers while DNA assisted cytology correctly identified 8 invasive cancers using the criterion that at least three cells were found on the slide that contained DNA amount in excess of 5c. DNA image cytometer correctly identified 30 cases out of 43 CIN2/ CIN3/CIS lesions versus only 23 by conventional cytology. The results of this study suggest that screening for cervical cancer by DNA image cytometer is more sensitive than conventional cytology in detecting high grade CIN lesions and cervical cancer although at the expense of a slightly reduced specificity (86.4% *vs.* 81.5%). Cytology combined with DNA image cytometer was also raised more sensitive than DNA image cytometer only.

It could be argued that in this study the conventional cytology was not at the level of a typical cytology laboratory. Kok *et al.* [5] shown that only 23 cases from 71 (~33% sensitivity) invasive cancer patients were suspected as carcinoma by cytology, the results comparable to those reported in this study. The performance of several laboratories was summarized by van der Graaf *et al.* [7] who reported that only about half of biopsy-documented invasive cancers were appropriately recognized by cytology of cervical smears.

In this work, cells with DNA amount greater than 5 c were used as the sole indicator of potential presence of cancer [13]. There is a large body of literature that suggested that cervical cancer and high grade lesions are associated with aneuploid population of cells [16]. These are readily detected if sufficient number of cells could be measured and when implemented and tested could improve the performance of this approach.

Cytology combined with DNA image cytometer could increase sensitive in detecting high grade CIN lesions and cervical cancer compared to conventional cytology. It is also suggested DNA image cytometer can be used to cervical cancer screening programs.

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Photothermal Therapy Using TiO_2 Nanotubes in Combination with Near-Infrared Laser

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ABSTRACT

We report the in vitro cell test and in vivo animal test results of titanium oxide nanotubes (TiO_2 NTs) as a potential therapeutic agent used for cancer thermotherapy in combination with near-infrared (NIR) laser. The in vitro cell test results show that both the cells exposed to NIR laser without TiO_2 NTs treatment and the cells treated with TiO_2 NTs but not with NIR irradiation had cell viabilities higher than 96%. Combination of these two techniques, however, shows cell viability less than 1%. The cell death rate strongly depended on the concentration of TiO_2 NTs. Also, the cell deaths were mostly due to necrosis but partly due to late apoptosis. The in vivo animal test results show that tumor cells can be completely destroyed without nearly giving damage to surrounding healthy cells by an injection of an adequate amount of TiO_2 NTs/NaCl suspension and a subsequent single continuous laser treatment at a moderately low laser illumination intensity for the exposure time optimized for the tumor size. These results suggest that TiO_2 NTs can be effectively utilized as a therapeutic agent for cancer thermotherapy due to their excellent photothermal property and high biocompatibility.

Keywords*: *Thermotherapy, TiO_2 Nanotubes, In Vitro Cell Test, In Vivo Animal Test, NIR

1. Introduction

In recent years, cancer thermotherapy techniques based on inorganic nanomaterials and NIR laser have attracted significant interest owing to their advantages over conventional surgical treatments. The inorganic nanomaterials currently demonstrated as thermal coupling agents in thermotherapy are gold nanoparticles (Au NPs) [1], gold nanorods [2], gold nanoshells [3], gold nanocages [4], gold nanocrystals [5], single wall carbon nanotubes (SWCNTs) [6], and porous silicon [7]. The advantages of thermotherapy include the anticipated reduction in morbidity and mortality, low cost, suitability for real-time imaging guidance, and the ability to perform ablative procedures on outpatients because of its non-invasive nature [3].

For irreversible destruction of cancer cells heat treatment at a temperature higher than 46℃ for more than 60 min is required in thermotherapy, but increasing the treatment temperature can shorten the time necessary to induce cytotoxicity [8]. In the conventional thermotherapies based on simple heating [9] most treatment failures result from insufficient temperature rises in the tumor tissues. Therefore, it is very important to develop a thermal coupling agent for thermotherapy with high photothermal effect in order to secure irreversible destruction of tumor cells in a short time without damaging adjacent healthy cells in thermotherapy. In this paper, we report the in vivo animal test results of TiO_2 NTs in combination with NIR light. TiO_2 is a highly functional material that has numerous interesting properties. As regards biomedical applications of TiO_2 NTs, their use in drug delivery applications have been reported recently [10,11]. However, application of TiO_2 NTs to cancer photothermotherapy has not been reported before. The thermotherapy based on TiO_2 NTs in combination with NIR laser is anticipated to be effectively utilized to cure various cancers such as cancer of the esophagus, gastric cancer, cancer of the colon, cancer of the skin, breast cancer, and liver cancer due to the excellent photothermal property as well as the high biocompatibility of TiO_2 NTs [12-16].

2. Methods

TiO_2 NTs samples used in the experiments were in a

state of fragments. First, TiO_2 NTs layers were formed by electrochemical anodization [17] of Ti thin foils in an electrolyte consisting of 0.3 wt.% NH_4F and 2 vol.% H_2O in ethylene glycol at 60 V for 17 h. The inner diameter of the TiO_2 NTs is ~ 100 nm and the thickness of the TiO_2 NTs layers is ~ 160 μm (**Figure 1**). Next, the TiO_2 NT layers were fragmented into small pieces with sizes less than 220 nm by using an ultrasonicator (Model: 2510E-DTH, BRANSON Ultrasonics Corp., USA) and then filtered through a 220 nm microfilter (Model: SLGV 033 RS, Millipore, USA). TiO_2 NTs/NaCl suspensions were prepared by mixing TiO_2 NTs with 9% saline solution and then ultrasonicating them for 6 h and finally filtering them with a 220 nm filter (model: MILLEX® - GV, Millipore, USA).

Annexin V-fluorescein isothiocyanate (FITC) apoptosis assays were performed on five different mouse CT-26 cell sample groups to see their modes of cell deaths: the CT-26 cell control group given neither TiO_2 NTs nor laser treatment, the CT-26 cell group not treated with TiO_2 NTs but with laser, the group not treated with laser but with TiO_2 NTs, the group treated with both a low concentration (50 mg/4 ml)-TiO_2 NTs/NaCl suspension and laser, and the group treated with both a high concentration (210 mg/4 ml)-TiO_2 NTs/NaCl suspension and laser to distinguish between apoptosis and necrosis. For Annexin V- FITC Apoptosis assay, the TiO_2 NT layer formed by the electrochemical anodization of a Ti thin foil was fragmented into fine particles by ultrasonicated for 24 h in a beaker filled with ethanol and then filtered through a 220 nm filter, first. After evaporation of the ethanol, TiO_2/NaCl:PEG suspensions with two different concentrations (50 mg/4 ml and 210 mg/4 ml) were prepared by mixing the TiO_2 particles with an 1:1 NaCl:PEG solution. Next, CT-26 cells were treated with one of the two TiO_2/NaCl: PEG suspensions and then

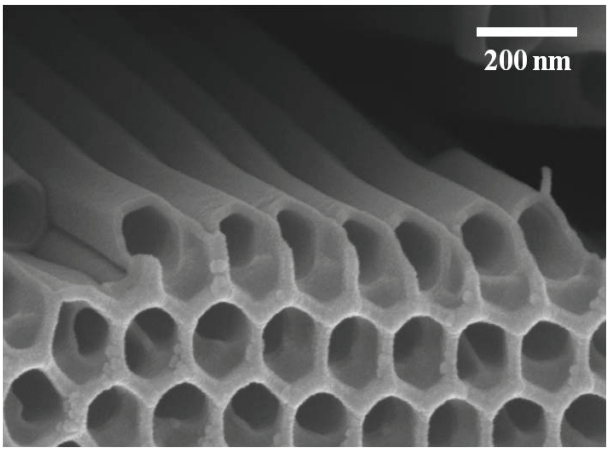


Figure 1. Bird's eye view SEM images of TiO_2 NTs. TiO_2 NTs were formed by anodic etching of Ti thin foils in an electrolyte consisting of 0.3 wt.% NH_4F and 2 vol.% H_2O in ethylene glycol at 60 V for 17 h

NIR laser at 300 mW/cm^2 for 20 min, 2×10^6 cells were removed from the culture, washed twice with cold PBS, and double stained with Annexin V-FITC and Propidium iodide (PI) (BD Biosciences, San Jose, CA, USA) in Annexin-binding buffer, followed by analysis on a FAC-Scalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488-nm argon laser. To avoid nonspecific fluorescence from dead cells, live cells were gated using forward and side scatter.

Animal care and all experimental procedures were conducted in accordance with 'Laws and regulations on animal experiments' [18]. In vivo animal tests were performed on 15 healthy BALB/C male mice, 6-7 weeks old, weighing 28-31 g. CT-26 murine colon carcinoma tumor cells were inoculated into the hollow of the back to transplant them to the mice. After the inoculation the mice were maintained in separate cages and fed with standard laboratory food and water. The tumors grew to ~ 1.0 cm in diameter within a week of the tumor transplant. Details of treatment procedures (TiO_2 NTs/NaCl suspension injection and then laser treatment) are as follows: The mice were anesthetized by injecting 40 μl of a 9: 1 solution of ketamine (100 mg/ml) and rompun (100 mg/ml), 24 h prior to laser treatment. The overlying skin was shaved. 100 μl of 27.5 or 52.5 mg/ml TiO_2 NTs/NaCl suspension was then directly injected ~ 7 mm into the tumor volume. NIR laser treatment was performed at 300 or 400 mW/cm^2 for 20 min. The treatment (the suspension injection plus the subsequent laser treatment) was repeated for 2 or 3 times with a time interval of 48 h between the treatments. The control group received no injection or subsequent laser treatment. Details of the parameters in the suspension injection and the laser treatment are listed in **Table 2**. The first, second, and fourth groups in **Table 1** were given the treatment (the suspension injection and the laser treatment) twice under the condition given to each group and the third given the treatment for three times in a similar manner. On the other hand, the fifth was given a single continuous laser treatment for 60 min.

3. Results and Discussion

The fluorescent activated cell sorter (FACS) flow cytometry profiles (**Figures 2(a)** and **(b)**) obtained as a result of Annexin V-FITC Apoptosis assay represent Annexin V-FITC staining in X-axis and PI in Y-axis. The four sections of the quadrant in each profile from the upper left in a clockwise direction represent necrosis, late apoptosis, early apoptosis, and live cell, respectively. The groups treated with TiO_2 NTs/NaCl suspensions show substantially higher cell death (necrosis + late apoptosis) rates than those not given both treatments (**Figures 2(a)** and **(b)**). The Annexin V-FITC Apoptosis assay results in (**Figures 2(a)** and **(b)**) are summarized in **Table 1**. The figure show that the cell death rate strongly depends on

Table 1. A summary of the Annexin V-FITC Apoptosis assay results shown in Figure 2, showing the percentages of cell death modes: necrosis, late apoptosis, early apoptosis, and live cell

	control	Only NIR	Only TiO_2	50 mg / 4 mL NaCl + NIR	210 mg / 4 mL NaCl + NIR
Necrosis	1.51	1.87	0.98	48.41	91.58
late apoptosis	0.05	0.27	0.55	1.00	7.07
early apoptosis	0.02	0.11	2.08	49.82	1.35
live cell	98.42	97.75	96.39	0.77	0.00

Table 2. Parameters in TiO_2 NTs / NaCl suspension injection and NIR laser treatment

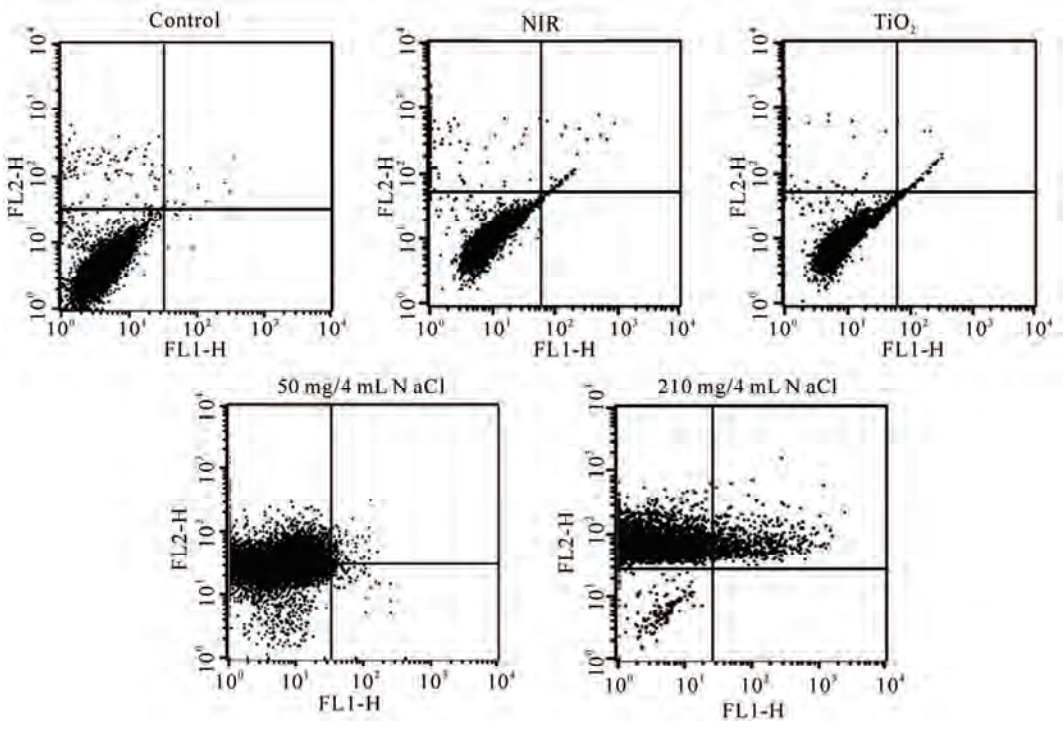
Laser intensity (mW/cm^2)	Exposure time (min)	TiO_2 NTs concentration in TiO_2 NTs/NaCl suspension (mg/ml)	No. of mice treated	Photograph	Average dia. of destructed volume (mm)
300	20 + 20	52.5	3	Figure 3(a)	4.4
400	20 + 20	52.5	3	Figure 3(b)	6.5
300	20 + 20 + 20	52.5	3	Figure 3(c)	5.2
300	20 + 20	27.5	3	Figure 3(d)	2.7
300	60	52.5	3	Figure 3(e)	4.3
None	0	0	3	Figure 3(f)	0
Total			18		

the concentration of TiO_2 NTs. The group treated with a high concentration (210 mg/4 ml)-TiO_2 NT suspension and exposed to NIR laser at 300 mW/cm^2 for 20 min without TiO_2 NTs treatment shows a cell viability of 97.8%. Likewise, the group treated with a high concentration (210 mg/4 ml)-TiO_2 NT suspension but not with NIR irradiation also shows a cell viability of 96.4% (**Figure 3**). Combination of these two techniques, however, shows cell viabilities of 0.77 and 0.0% for lower (50 mg/4 ml) and higher (210 mg/4 ml) TiO_2 NT concentrations, respectively, implying that most cells are killed. The cell deaths are mostly due to necrosis but partly due to late apoptosis. The in vitro cell test results suggest that only combination of both TiO_2 NTs and NIR laser treatments can kill cells and that a higher TiO_2 NT concentration results in a higher cell death rate, particularly a higher necrosis rate than a lower TiO_2 NT concentration.

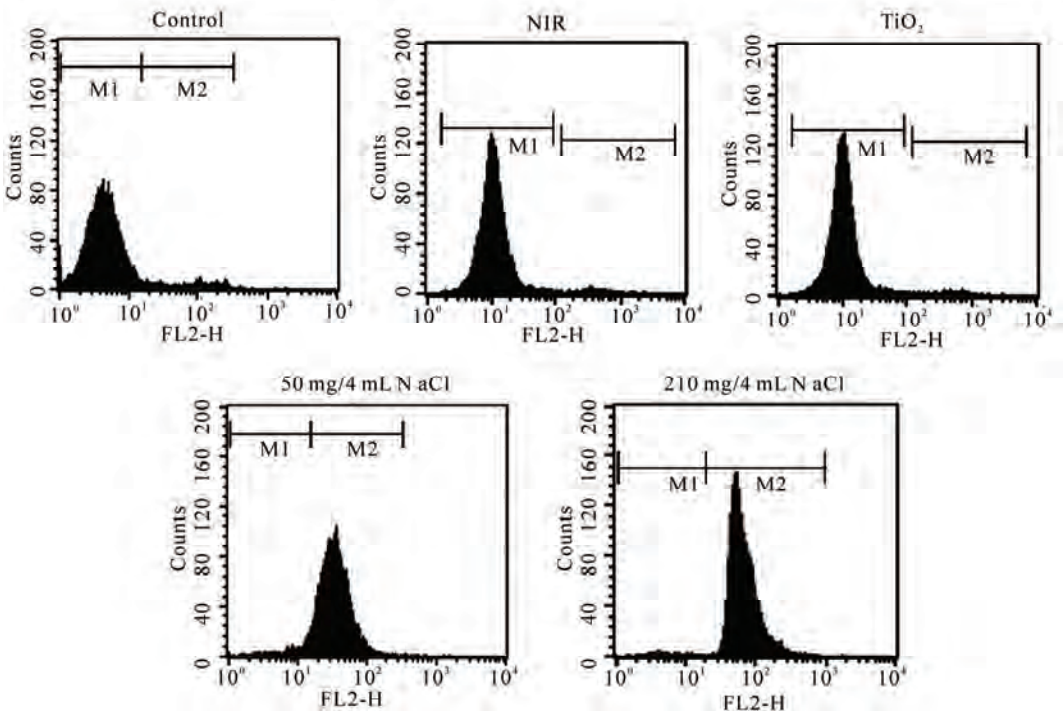
The in vitro cell test results show the photothermal effect of the thermotherapy based on TiO_2 NTs combined with laser on cell death, but it does not guarantee that the thermotherapy can inhibit tumor growth. Therefore, we conducted in vivo animal tests to confirm that the photothermal effect of TiO_2 NTs combined with NIR laser could efficiently destroy tumor cells without giving damage to surrounding healthy cells and to investigate the influence of the treatment parameters including the amount and concentration of the suspension, the laser illumination intensity, and the laser exposure time on the destruction efficiency of tumors.

All the mouse CT-26 tumors used in this study were examined and euthanized 48 and 96 h after the last laser treatment, respectively. We chose 48 h after the last treatment as a point of time for examining the photothermal damage since necrosis of the tumor cells by the photothermal effect has usually continued for one or two days after laser treatment. First, we tried a single treatment (for example, an injection of 100 μl of the suspension with a TiO_2 NTs concentration of 52.5 mg/ml into tumors and a subsequent laser treatment at 300 mW/cm^2 for 20 min), but the photothermal effect by the treatment was lower than we expected. Hence, we decided to repeat treatment twice.

The average diameters and photographs of the tumors 48 h post-treatment of the six mouse tumor groups given different treatments are shown in **Table 1** and **Figures 3(a)-(f)**, respectively. The injured areas look black owing to carbonization of the skin tissue as well as the tumor tissue by the photothermal energy generated during laser treatment by the TiO_2 NTs/NaCl suspension injected into the tumors. The tumor grew quite a bit after laser treatment (**Figure 3(a)**) compared with control (**Figure 3(f)**) due to incomplete destruction of tumor cells and rapid



(a)



(b)

Figure 2. FACS flow cytometry profiles ((a) dot plots and (b) histograms) obtained as a result of Annexin V-FITC Apoptosis assay for five different mouse CT-26 cell sample groups to see their modes of cell deaths: the CT-26 cell control group, the CT-26 cell group treated with laser, the group treated with TiO_2 NTs, the group treated with both TiO_2 NTs (50 mg/4 ml) and laser, and the group treated with both TiO_2 NTs (210 mg/4 ml) and laser. The NIR laser irradiation intensity and time in the treatments were 300 mW/cm^2 and 20 min, respectively

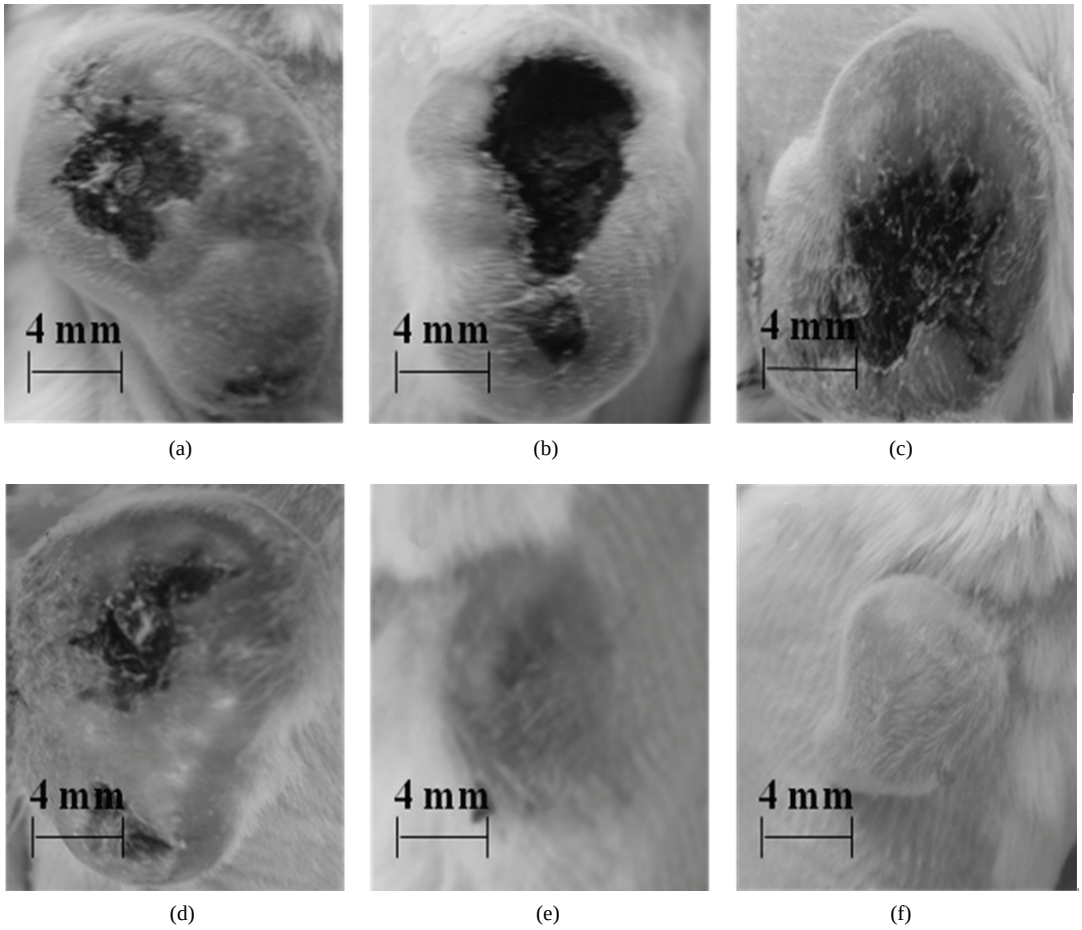


Figure 3. Photographs of the tumors of mice 48 h after treatment : (a) double laser treatments (laser illumination intensity = 300 mW cm^{-2}, exposure time = 20 min, TiO_2 NTs concentration = 52.5 mg ml^{-1}), (b) double laser treatments (400 mW cm^{-2}, 20 min, 52.5 mg ml^{-1}), (c) triple laser treatments (300 mW cm^{-2}, 20 min, 52.5 mg ml^{-1}, (d) double laser treatments (300 mW cm^{-2}, 20 min, 27.5 mg ml^{-1}), and (e) single laser treatment (300 mW cm^{-2}, 60 min, 52.5 mg ml^{-1}), (f) no laser treatment (control)

growth of survived cancer cells. It appears that only a part of the tumor was destroyed since the laser treatment time of 20 min per treatment was sufficiently long. Comparison of **Figure 3(b)** with **Figure 3(a)** reveals that photothermal damage on tumor cells strongly depends on the intensity of the laser delivered. The average diameter (6.5 nm) of the destroyed volume in the tumor samples given a suspension injection and a subsequent laser treatment at a higher illumination intensity is considerably larger than that (4.4 nm) in those given a similar treatment at a lower intensity. Comparing **Figure 3(c)** with **Figure 3(a)**, we can also see strong dependence of the photothermal destruction efficiency of tumor cells on the number of treatments or the total laser exposure time although the influence of the laser treatment time on the tumor destruction is not as strong as that of the laser illumination intensity. The average diameter of the damaged area (5.2 mm) in the tumor samples given a treatment (a suspension injection plus a subsequent laser treatment at 300 m/cm^2) for three times is somewhat larger than that (4.4 mm) in the tumor samples given the same treatment twice. On the other hand, it can be seen by a comparison of **Figure 3(d)** with **Figure 3(a)** that the difference in the TiO_2 NTs concentration of the TiO_2 NTs/NaCl suspension also led to a clear distinction in the photothermal damage on tumor cells. The average size of the tumor samples given a treatment with a higher concentration is about 1.6 times as large as that of the tumor samples given a similar treatment with a lower concentration.

All the tumors given a double treatment in **Table 1** were incompletely destroyed (**Figures 3(a)-(d)**) and have grown much faster than those in the control group (**Figure 3(f)**) after each treatment. In contrast, two of the three samples in the tumor group given a suspension injection plus a subsequent 60 min laser treatment, i.e. a long single continuous treatment (**Figure 3(e)**) were so perfectly destroyed that they did not grow at all after the laser treatment and their skin tissue was not nearly damaged in spite of the perfect death of their tumor cells.

One of those given the same treatment grew due to incomplete destruction, but its skin tissue was not damaged like those of the other two in the same group. **Figure 3(e)** shows the intact surface structures and the size of one of the two tumors, whose cells were completely killed, nearly unchanged in comparison with that at the time of laser treatment (**Figure 3(f)**). By comparing **Figure 3(f)** with **Figures 3(a)-(d)**, we can clearly see that a long single continuous laser treatment is most effective in destroying tumor cells since it can stop the growth of the tumors without giving damage at all to the skin tissue.

The carbonization of the tissue including the epidermis and subcutaneous tissue overlying the tumors in the tumor samples given a double treatment (**Figures 3(a)-(d)**) is caused by the reaction of the elements such as carbon, hydrogen, and oxygen comprised in tissue with oxygen molecules in air. It appears that the carbonization of the skin tissue initiated at a localized surface site where TiO_2 NT fragments happened to migrate due to the large amount of suspension (100 μl × 2) injected into the tumor and then to expand to neighbors. Once a part of the tumor surface is carbonized, carbonization of the tumor cells is accelerated since the carbonized tissue is an ideal absorber of the NIR light [19]. In contrast to double treatments (**Figure 3(a)-(d)**), the skin tissue of the tumor given a long single treatment (**Figure 3(e)**) was not damaged at all since there were less chance of migration of TiO_2 NT fragments to the tumor surface due to the smaller amount of suspension (100 μl × 1) in the tumor, but all the tumor cells appears to have been killed since the laser treatment time was sufficiently long. This result suggests that a long single treatment with a smaller total amount of suspension is more efficient in killing tumor cells without injuring adjacent healthy cells than a multiple treatment with a larger total amount of suspension. It is also worthy of noting that all the mice employed in this experiment were alive until the time of euthanasia, suggesting that all those four parameters were properly chosen in safe ranges.

4. Conclusions

The in vitro cell test results show that the cells exposed to NIR laser without TiO_2 NTs treatment have a cell viability of 97.8%. Likewise, the cells treated with TiO_2 NTs but not with NIR irradiation also have a cell viability of 96.4%. Combination of these two techniques, however, shows a cell viability of 0.0%. The cell death rate strongly depends on the concentration of TiO_2 NTs. Also, the cell deaths are mostly due to necrosis but partly due to late apoptosis.

According to the in vivo animal test results the photothermal destruction efficiency of tumor cells can be enhanced by increasing any of the four parameters used in this study, namely, the laser intensity, the laser exposure time, the amount of the TiO_2 NTs suspension and the TiO_2 NTs concentration, yet excessive increases in these parameters may result in destruction of not only the tumor tissue but also the surrounding tissue including the epidermis and subcutaneous tissue. Therefore, the exposure time should be optimized under the condition of a moderately small amount of suspension, a moderately low concentration of suspension and a moderately low laser intensity in order to destroy all the tumor cells without giving damage to surrounding healthy tissue. It is also worthy of noting that all the mice employed in this experiment were alive until the time of euthanasia, suggesting that all those four parameters were properly selected in safe ranges. These results suggest that TiO_2 NTs can be used effectively as therapeutic agents for cancer thermotherapy owing to their excellent photothermal properties and high biocompatibility.

5. Acknowledgements

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Experimental Study of the Antitumor Activity of Polymetalacrylates against Animal Transplantable Tumors

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ABSTRACT

The antitumor activity of the fourteen polymetalacrylates against two models of murine solid tumors (*Lewis lung carcinoma and Acatol adenocarcinoma*) *as well as the acute toxicity of these compounds has been studied. It was shown that polyacrylates of noble metals* (*argent, aurum, platinum*), *namely argacryl* (*M = Ag*), *auracryl* (*M = Au*) *and platacryl* (*M = Pt*) *were the most effective agents among tested compounds against studied tumors. Thus, the tumor growth inhibitory effect of argacryl against Lewis lung carcinoma was equal to* 90%, *the life-span of treated by this compound animals has increased on* 50% *in comparison with control. Auracryl induced the inhibition of the Lewis lung carcinoma and Acatol adenocarcinoma development on* 60 *and* 65%, *correspondingly and the increasing of the mean life-span of animals with Lewis lung carcinoma on* 20% *in comparison with control. Platacryl inhibited the growth of Lewis lung carcinoma on* 40% *increasing the mean life-span of animals on* 25% *in comparison with control. In this way it was established that argacryl is the agent with the strongest antitumor activity among studied polymetalacrylates. On the basis of obtained data it seems possible to consider polymetalacrylates as a group of agents with the potential antitumor activity suitable for the further deep experimental investigation.*

Keywords*: *Polymetalcrylates, Antitumor Activity, Transplantable Tumors of Animals

1. Introduction

The development of drugs capable of inhibiting the growth of a malignant tumor at the expense of the influence on its blood supply is one of recognized areas in the field of biomedical chemistry and experimental oncology. Modern approaches to the solution of this problem are based on the investigation of medicines causing regional embolism of vessels, feeding a tumor, or creation of drugs inhibiting neo-angiogenesis processes in tumor. Over 500 various chemical compounds possessing an anti-angiogenesis activity against some experimental models are known at the present time [1].

In this field, the metal derivatives of polyacrylic acid (polymetalacrylates) are especially promising [2-9].

A pharmacological mechanism of action of polymetalacrylates is based first of all on their unusual influence on a coagulating system of blood leading to the formation of interpolymers with blood plasma proteins [10,11].

The first representative of medicines of this type, feracryl, is allowed for a broad medical use in Russia as a blood-stopping agent [12-16]. Argacryl (an incomplete silver salt of polyacrylic asid) is later offered as an effective hemostatic with a broad spectrum of pharmacological properties [2-4]. Silver containing medicines (poviargol, protargol, kollargol, argovit etc.) are successfully used in medical practice as antiseptic remedies actively suppressing a pathogenic flora and simultaneously stimulating an immune status [17].

It is well known that drugs containing complexes of metals of platinum group (cisplatin, carboplatin *et al.*) are one of the most commonly used cytostatics in the modern antitumor chemotherapy [1,18-20].

Metallic derivatives of polyacrylic acid were not previously tested as antitumor agents.

The aim of our research has been the study of the antitumor activity of polymetalacrylate derivatives containing in their structure different metals (PMA) for the

reveal of the most effective compounds interesting for a further deep experimental investigation.

2. Materials and Methods

2.1 Compounds

The fourteen metal derivatives of polyacrylic acid have been studied in the biological experiment. These incomplete metallic salts of polyacrylic acid containing metal ions (**Table 1**) correspond to the general formula $(\text{-CH}_2\text{-CH-COOH})_n(\text{-CH}_2\text{-CH-COOM})_m$, where n = 1200-3500; m = 1650-6650 and contain 4-8 mass% of a metal are colorless or colored films. They have been synthesized by the method of polymer analogous transformations of polyacrylic acid by organic and inorganic metal salts [2-9]. The molecular weight of these polymetalacrylates are in the range from 1000000 to 3000000 D. Absorbtion bands 1548-1540 cm^{-1} (ν_{as} COO^-) и 1405-1410 cm^{-1} (ν_s COO^-); 1694-1649 cm^{-1} (COOH), a widened band in the region 3420-2554 cm^{-1}, shifted in a low-frequency field (associated OH).

Compounds prepared on the ground of PMA were used as aqueous solutions injected intraperitoneally (i/p) to experimental animals (0.2-0.8 ml). Under the identifycation of parameters of the acute toxicity - the mean lethal dose result to the death of 50% of animals (LD_{50}) and the maximum tolerated dose not causing the death of any mice (MTD)-agents were injected one time, i/p, in the wide range of doses (from 10 to 200 mg/kg).

For the estimation of the antitumor effect, compounds were injected i/p, five times, every day starting from the next day after tumor transplantation. Daily doses for the most of agents were closed to 1/5 from MTD, but the ones for polyacrylates of noble metals (argacryl, auracryl and platacryl) were closed to 1/10 from MTD.

2.2 Laboratory Animals

Experiments have been carried out on 400 inbred mice BDF_1-first generation hybrids $f_1(DBA_2 \times C_{57}Bl/_6)$ and Balb/c, males with the body weight of 18-20 g (Nursery "Stolbovaya" of the Russian Academy of Medical Sciences). The animals feeding regimen was based on the usage of the standard certificated commercial dry extruded food for rodents (PK-120-1) [21].

2.3 Experimental Models

Solid tumors, such as Lewis lung carcinoma and Acatol adenocarcinoma have been used as experimental test-systems. The transplantation of tumors has been done according to the standard procedures, subcutaneously at the right flank of mice by fragments of a tumor tissue suspended in a physiological solution of sodium chloride. The size of the inoculation material was equal to 0.3 ml [21].

2.4 The Antitumor Activity Test

Kinetics of the tumor growth in groups of treated (T) and control (**C**) animals as well as duration of mice life-span in both groups were studied. The coefficient of the tumor growth inhibition (TGI, %) and the increasing of the mean life-span (Δτ, %) of treated animals in compare with control were the indicators of the antitumor activity of tested drugs. The TGI coefficient has been determined according to the correlation: TGI = $\mathbf{(P_C - P_T)/P_C\%}$, where $\mathbf{P_C}$ and $\mathbf{P_T}$ are volumes (or weights) of tumor in the control group and group of treated animals, accordingly. Two mutually perpendicular sizes of the tumor node were measured over the whole period of tumors development for the study of the kinetics of the tumor growth. The tumor volume was measured according to the formula for an ellipsoid as $\mathbf{V = ab^2/2}$, where **a** is the length, **b** is the width and height of a tumor. The tumor weight corresponds to its volume, since the density of a tumor tissue is generally agreed to be equal to 1 g/cm^3 [21].The increasing of the mean life-span (**Δτ,%**) of treated animals in comparison with the control was evaluated as $\mathbf{\Delta\tau = (\tau_C - \tau_T)/\tau_C}$, %, where $\boldsymbol{\tau_C}$ and $\boldsymbol{\tau_T}$ are the mean life-span of control and treated animals, accordingly.It is assumed to consider as minimal significant the value of TGI coefficient equal to 50% as well as the value of the increasing of the mean life-span (**Δτ**) of treated animals in comparison with the control equal to 25% [21]. In accordance with the standard recommendations each group of treated and control animals comprised six and eight mice, correspondingly [21]. Experiments were repeated two or three times. The results of the main experiments are represented as kinetic curves of the tumor growth in groups of treated and control animals. Each dot on these curves presents the mean value of the tumor weight for 12-18 mice.

Experimental animals were observed during the whole period of the development of tumors.

Computer program "Statistics 6.0" was used for the statistical analysis of experimental data. F-criterion significance test was used for the estimation of the confidence of difference between mean values of tumor weight in groups of treated and control animals. It is consider that difference between values of tumor weight in groups of treated and control animals is confidence if the estimated value "F" is more than the value of "F-criterion" known for prescribed level of significance and for the certain degrees of freedom (f_1; f_2) [22].

3. Results

The fourteen polimetalacrylates (PMA) including four compounds containing noble metals, such as silver, gold, platinum, palladium, have been studied in biological experiments. Acute toxicity of PMA has been defined. The antitumor activity of compounds against Lewis lung car-

cinoma and Acatol adenocarcinoma has been established.

3.1 Toxicity

All studied compounds have a good solubility in water (excluding poorly soluble palladacryl).

When studying the compounds acute toxicity it has been established that PMA LD_{50} values vary within the limits from 15 to 200 mg/kg, while values of MTD vary in diapasons from 10 to 150 mg/kg depending from the nature of the metal (M).

From the data indicated in the **Table 1** it follows that auracryl (M = Au) and feracryl (M = Fe) have a lower order toxicity than the other studied polymetalacrylates. LD_{50} and MTD for auracryl were equal to such values, as 150 and 100 mg/kg, the same ones for feracryl were equal to 200 and 150 mg/kg correspondingly. The same indicators for two other derivatives containing noble metals-platacryl (M = Pt) and argacryl (M = Ag) were equal to 75; 50 mg/kg and 25; 20 mg/kg respectively (**Table 1**).

The values of the LD_{50} for the other ten compounds were varied in the range from 15 till 50 mg/kg. The values of the MTD for these compounds were varied in the range from 10 till 40 mg/kg (**Table 1**).

So as it is seen from the data represented in the **Table 1** the most toxic agents between tested compounds were derivatives containing such metals as cesium (Cesacryl), nickel (Nicacryl), stannum (Stanacryl), while the less toxic one was the ferrum derivative (Feracryl).

Table 1. The acute toxicity of polymetalacrylates (the single i/p administration of agents, BDF_1 mice)

# #	PMA	Metal	The maximum tolerated dose (MTD) mg/kg	The mean lethal dose (LD_{50}) mg/kg
1	Liacryl	Li	40	50
2	Rubacryl	Rb	20	30
3	Cesacryl	Cs	10	20
4	Licuacryl	Li+Cu	20	25
5	Mangacryl	Mn	30	35
6	Feracryl	Fe	150	200
7	Nicacryl	Ni	10	15
8	Palladacryl	Pd	10	not defined
9	Platacryl	Pt	50	75
10	Argacryl	Ag	20	25
11	Auracryl	Au	100	150
12	Ziacryl	Zn	15	20
13	Cadacryl	Cd	30	35
14	Stanacryl	Sn	10	15

3.2 Antitumor Activity

The antitumor activity of PMA against Lewis lung carcinoma and Acatol adenocarcinoma was tested.

The sensitivity of Lewis lung carcinoma to PMA compounds are characterized by data represented in **Tables 2**, **3** and on the **Figure 1**.

As it is seen from data below argacryl, auracryl and platacryl are the most effective among studied polymetalacrylates against this tumor model.

Thus, the tumor growth inhibitory effect of argacryl against Lewis lung carcinoma this tumor was equal to 90%. The life-span of treated by argacryl animals has increased on 50 % in comparison with control (**Tables 2**, **3**; **Figure 1**).

In case of auracryl the coefficient of the tumor growth inhibition was equal to 60% and the increase of the mean life-span of treated animals was 20% in comparison with control (**Tables 2**, **3**; **Figure 1**).

Platacryl inhibits the growth of Lewis lung carcinoma on 40% increasing the mean life-span of treated animals on 25% in comparison with control (**Tables 2**, **3**; **Figure 1**).

Asaturated aqueous solution of palladacryl containing less than 2% of palladium does not inhibit the growth of the tumor studied (**Tables 2**, **3**).

It should be mentioned that some tested compounds such as cadacryl, rubacryl, feracryl induced the increasing of the mean life-span of treated mice with Lewis lung carcinoma on 53, 39 and 31% in compare to control correspondingly, but didn't influence on the rate of tumors growth (**Tables 2**, **3**).

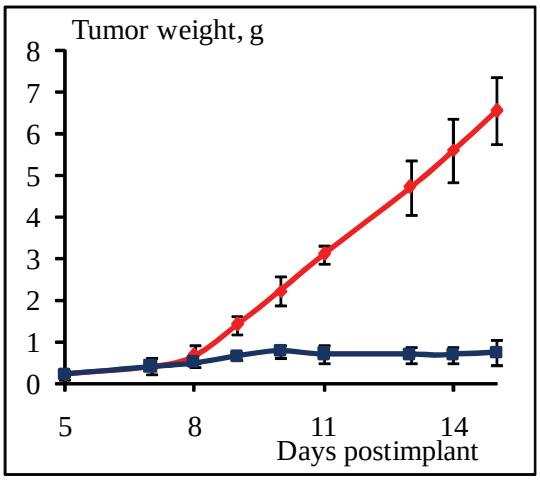
The obtained results prove that derivatives of noble metals (M = Ag, Au, Pt), espesially argacryl, are the most effective among studied polymetalacrylates against Lewis lung carcinoma.

The development of the other studied tumor - Acatol adenocarcinoma was the most effectively inhibited by auracryl – the value of the TGI coefficient for this agent was equal to 65% (**Table 4**, **Figure 2**).

Platacryl and argacryl inhibited the growth of Acatol adenocarcinoma on 55% and 50% in comparison with control, accordingly (**Table 4**, **Figure 2**).

Therefore, the results of these experiments prove that compounds argacryl, auracryl and platacryl pronounced the significant antitumor effect against some murine solid tumors such as Lewis lung carcinoma and Acatol adenocarcinoma.

The received data allows to consider polymetalacrylates as a new group of compounds with a potential antitumor activity. Antitumor effect of polymetalacrylates containing noble metals (Ag, Au, Pt) has been first estab-



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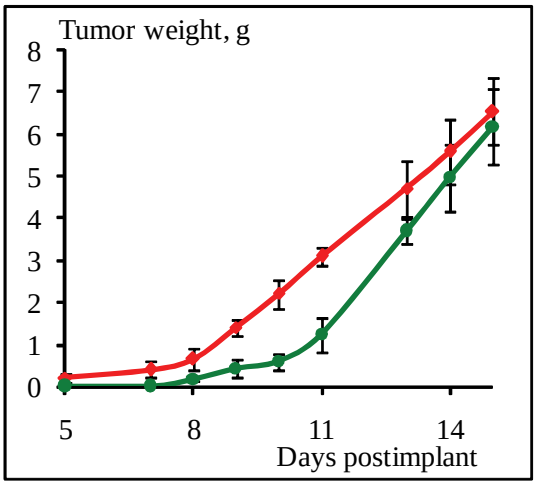

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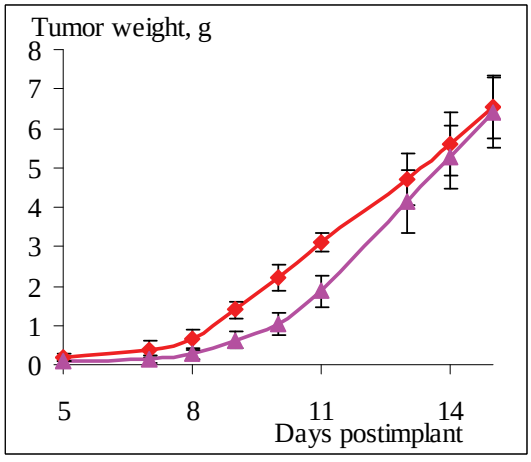
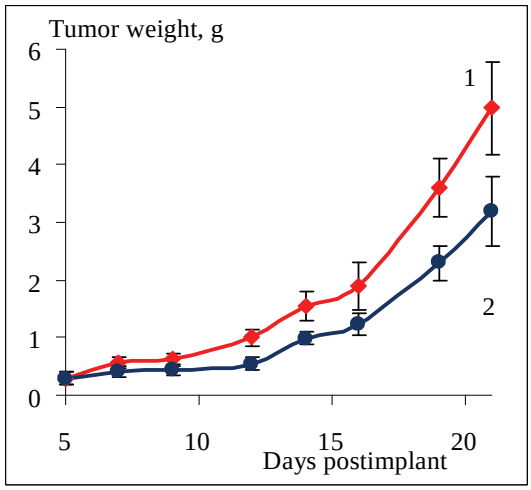
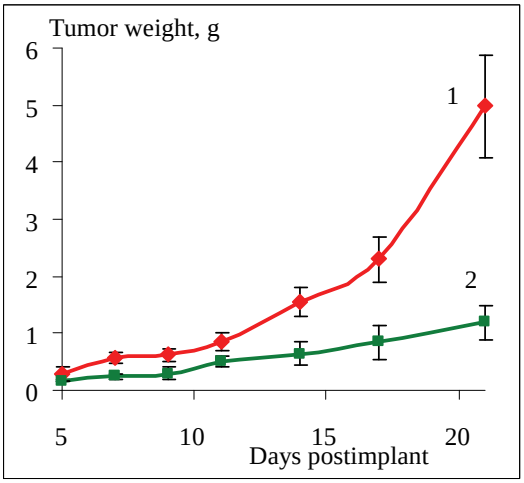


(c)

Figure 1. Antitumor activity of Argacryl (a) Auracryl, (b) Platacryl, (c) against Lewis lung carcinoma(intraperitoneal administration of compounds, 5 times, days 1-5); 1—control, 2—corresponding compound; Argacryl—2 mg/kg/day, Auracryl—10 mg/kg/day, Platacryl—4 mg/kg/day



(a)



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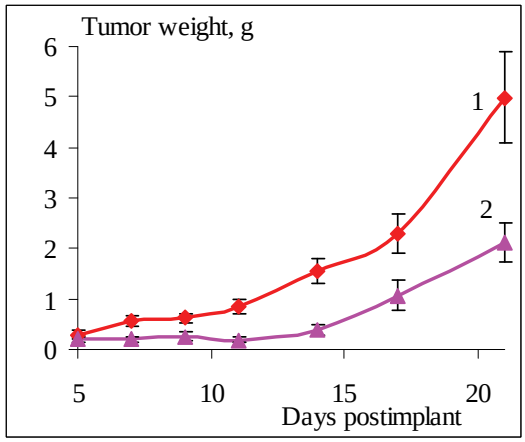

(c)

Figure 2. Antitumor activity of Argacryl (a) Auracryl, (b) Platacryl, (b) against Acatol adenocarcinoma (intraperitoneal administration of compounds, 5 times, days 1-5); 1—control, 2—corresponding compound; Argacryl—1 mg/kg/day, Auracryl—10 mg/kg/day, Platacryl—4 mg/kg/day

Table 2. Antitumor activity of polymetalacrylates (PMA) against Lewis lung carcinoma (BDF_1 mice)

##	PMA	Daily dose (mg/kg)	The mean tumor weight (g) P ± SD	Day after tumor transplantation	The coefficient of the tumor growth inhibition TGI, %	$F_{0.001}$-criterion significance test (f_1=15;f_2=11)
1	Liacryl	10	6.5 ± 0.4	15	1	-
2	Rubacryl	2	4.3 ± 1.1	15	35	-
3	Cesacryl	2	6.6 ± 1.1	15	0	-
4	Licuacryl	4	5.0 ± 1.1	15	24	-
5	Mangacryl	5	6.6 ± 1.3	15	0	-
6	Feracryl	20	5.4 ± 0.8	15	18	-
7	Nicacryl	2	6.3 ± 1.9	15	5	-
8	Palladacryl	2	6.3 ± 1.4	15	5	-
9	**Platacryl*)**	**4**	**1.9 ± 0.3**	**11**	**40*)**	**5.44>4.25**
10	**Argacryl**	**2**	**0.7 ± 0.3**	**15**	**90**	**7.10>4.25**
11	**Auracryl*)**	**10**	**1.2 ± 0.3**	**11**	**60*)**	**5.44>4.25**
12	Ziacryl	1	5.4 ± 1.2	15	18	-
13	Cadacryl	5	4.6 ± 1.2	15	31	-
	Control***)**	-	3.1 ± 0.7	11	-	-
	Control	-	6.6 ± 0.8	15	-	-

Compounds were injected i/p, five times, every day starting from the next day after tumor transplantation.
***)** The coefficient of the tumor growth inhibition (TGI, %) for platacryl and auracryl was evaluated on the 11^{th} day after tumor transplantation, for all other agents - on the 15^{th} day after tumor transplantation.

Table 3. The influence of polymetalacrylates (PMA) upon the mean life-span (Δτ,%) of animals with Lewis lung carcinoma (BDF_1mice)

##	PMA	Daily dose (mg/kg)	The mean life-span of mice (days) τ ± SD	The change of the mean life-span of treated mice in compare to control (Δτ,%)
1	Liacryl	10	30.8 ± 5.9	23
2	Rubacryl	2	34.8 ± 2.1	39
3	Cesacryl	2	32.3 ± 1.5	29
4	Licuacryl	4	25.8 ±2.6	3
5	Mangacryl	5	30.2 ± 2.5	20
6	Feracryl	20	32.8 ± 4.5	31
7	Nicacryl	2	25.0 ± 5.8	0
8	Palladacryl	5	24.7 ± 2.5	0
9	**Platacryl**	**4**	**31.3 ± 4.2**	**25**
10	**Argacryl**	**2**	**36.5 ± 2.6**	**46**
11	**Auracryl**	**10**	**30.0 ± 4.3**	**20**
12	Ziacryl	1	24.8 ± 2.6	0
13	Cadacryl	5	38.3 ± 1.2	53
	Control	-	25.0 ± 2.8	- -

Table 4. Antitumor activity of polymetalacrylates against Acatol adenocarcinoma (Balb/c mice)

##	PMA	Daily dose (mg/kg)	The mean tumor weight (g) P±SD	The coefficient of the tumor growth inhibition TGI, %	$F_{0.001}$-criterion significance test ($f_1 = 15$; $f_2 = 11$)
1	Liacryl	10	3.6 ± 1.7	28	-
2	Rubacryl	2	3.2 ± 1.2	36	-
3	Cesacryl	2	4.4 ± 1.8	12	-
4	Licuacryl	4	4.1 ± 2.6	18	-
5	Mangacryl	5	4.7 ± 1.5	7	-
6	Feracryl	20	4.9 ± 1.5	1	-
7	Nicacryl	2	5.1 ± 1.2	0	-
8	Palladacryl	2	4.8 ± 0.6	0	-
9	**Platacryl**	**4**	**2.1 ± 0.4**	**58**	**5.06 > 4.25**
10	**Argacryl**	**2**	**3.2 ± 0.4**	**36**	**5.06 > 4.25**
11	**Auracryl**	**10**	**1.2 ± 0.3**	**76**	**9.00 > 4.25**
12	Ziacryl	1	3.5 ± 1.5	30	-
13	Cadacryl	5	5.2 ± 1.3	0	-
	Control	-	5.0 ± 0.9	-	-

Compounds were injected i/p, five times, every day starting from the next day after tumor transplantation.
The coefficient of the tumor growth inhibition TGI, % was evaluated on the 21th day after tumor transplantation.

lished. Thus, these compounds may be recommended for the further advanced experimental studies as potential antitumor agents.

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Platelet Endothelial Cell Adhesion Molecule (PECAM-1) Expression in Malignant Human Tumours and their Metastases

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ABSTRACT

PECAM-1 *is an adhesion molecule that plays an important role in the process of tumour disease dissemination since a function in transendothelial migration, angiogenesis and immune response has been shown for this membrane protein. Nevertheless the expression of PECAM*-1 *protein in solid tumours is a controversial matter and it has not been clarified so far. Thus, the aim of our study was to investigate PECAM*-1 *expression by immunohistochemistry in primary carcinomas from colon, breast, bladder, ovary and kidney, and in their metastases. In addition an example of primary and metastatic melanoma was also investigated. We found that PECAM-1 is expressed in the metastases of all primary carcinomas that express PECAM*-1 (*colorectal, breast and urothelial bladder*). *By the contrary metastases from primary carcinomas non-expressing PECAM*-1 *are also negative for expression. In conclusion, our findings support a possible role of this molecule in metastatic development of a subset of malignant human epithelial tumours.*

Keywords**:** *PECAM*-1, *CD*31, *Angiogenesis, Neoplasms, Immunohistochemistry*

1. Introduction

Metastatic dissemination is a complex biological process in which the tumoral cells escape immunological surveillance, migrate from its initial site through the vascular endothelium and finally grow up into a new tissue. The expression of adhesion molecules plays a key role in the process of metastases and neoangiogenesis.

PECAM-1 (CD31 or EndoCAM), a member of the immunoglobulin superfamily, is a membrane glycoprotein type 1 of 711 aminoacids [1] classified as an adhesion molecule [2]. It is a member of the inmunoglobulin super family that is encoded by a 75-kb gene that resides at the end of the long arm of chromosome 17 [3]. This glycoprotein is expressed in platelets, lymphocytes, monocytes, natural killer cells [4,5] and also in interendothelial junctions[6]. PECAM-1 has either homophilic [7,8] or heterophilic adhesion capacities with other molecules such as integrines [9,10] and glycosaminoglycanes [11]. Moreover, PECAM-1 trough its cytoplasmic domain transmit out-in signals from the cell surface to the nucleus [12,13]. In experimental inflammation models a functional inhibition of PECAM-1 with monoclonal antibodies prevents leukocyte migration to the inflammatory focus [14,15]. In addition, PECAM-1 also participates in the T-cell mediated activation in alloimmune response [16] and in natural killer cell activation, proliferation and migration [17]. Recent studies have demonstrated an even wider range of functions for CD31 including maintenance of adherens junction integrity and permeability, organization of the cytoskeleton, transcriptional activities, participation in STAT isoform signaling among others. [18]

PECAM-1 is also believed that participate in the neoangiogenesis process [6,19]. For instance, it has been suggested that PECAM-1 play an important role in the formation of new vessels, through homophilic interactions in the endothelial junctions, or the heterophilic adhesion the integrine $\alpha_v\beta_3$, which are crucial unions in endothelial cell migration through the extracellular matrix. [20]. To gain insight into the role PECAM-1 plays during vascular development and angiogenesis, for example it was examined the expression pattern of PECAM-1 isoforms during kidney vascularization showing

that regulated expression of specific PECAM-1 isoforms may enable endothelial cells to accommodate the different stages of angiogenesis [21].

PECAM-1 has been shown to potently suppress apoptosis in a variety of cellular systems, for example on a variety of human malignancies -especially hematopoietic and vascular cell cancers-. The ability of PECAM-1 to inhibit apoptosis makes it an attractive candidate as a molecule that may promote cancer development and/or confer resistance to chemotherapeutic treatment. In a recent study, it was shown that the endogenous PECAM-1 expression on lymphoid cancers confers resistance to apoptosis, and that lowering PECAM-1 expression in lymphoid malignancies can render them more susceptible to chemotherapy-induced apoptosis. [22,23]. The expressión of PECAM-1 in some hematopoietic malignancies has been studied and correlated with a worse prognosis, for example in a subgroup of patients with B-cell chronic lymphocytic leukemia (B-CLL) [24] or with primary non-Hodgkin's gastric lymphoma. [25].

Previous studies have shown that some cellular lines derived from solid tumours express PECAM-1 (Hep-1, MS751, TCC, MFC-7, DLD-1) and that anti-PECAM-1 monoclonal antibodies inhibit the tumoral cell adhesion to the endothelium "in vitro" [26]. Interestingly, it has been observed that in gliomas [27], breast carcinomas, osteosarcomas and in lymphomas [28] PECAM-1 expression is related to spread and disease progression. Moreover, PECAM-1 has also been used as a histological neoangiogenesis marker since a correlation between expression and prognosis has been demonstrated in breast cancer and melanomas [29,30]. The expression of PECAM-1 could be related to endothelial transdifferentiation of melanoma cells although a consequent functional role has not been demonstrated yet [31].

The functions in which PECAM-1 may participate (adhesion, transendothelial migration and neoangiogenesis) are key steps in metastatic dissemination. Therefore the study of PECAM-1 in primary and metastatic tumours is of special interest to understand the role of this antigen in the metastatic process. In the present report we have analysed PECAM-1 expression in a selected group of common solid human tumours both in primary and in metastatic localisation.

2. Material and Methods

Tissue samples from colon, breast, urinary bladder and kidney carcinomas and melanoma were selected to study the expression of PECAM-1 (**Table 1**). Surgical material was fixed in 4% phosphate-buffered formalin and paraffin embedded.

Selected 4-6 mms sections were utilized for immunohistochemistry using the avidin-biotin-peroxidase complex method. After deparaffination, unstained slides were treated with microwave heating for antigen retrieval solution (citrate buffer pH 6.0) for 12 minutes. The anti-PECAM-1 antibody (clone HC 1/6) (Cabañas, 1989) was applied at a 1:1 dilution, then followed by biotinilated anti-mouse Ig G secondary antibody and Vectastain ABC kit (Vector Laboratories Inc.). Diaminobenzidine (Sigma) was used as substrate, and the slides were slightly counterstained with hematoxylin. Negative controls were carried out in the absence of the specific antibody and

Table 1. Immunohistochemical expression of PECAM-1 in colon cancer, breast cancer and bladder cancer specimens

Identification	Localisation Tumour	Immunohisto-chemical expression	Localisation Metastases	Immunohisto-chemical expression
FCH5388/5342	Colorectal	+++	Liver	++
CMM670/671	Colorectal	++	Liver	++
MRL6638/6640	Colorectal	+++	Liver	+++
ARM17997/17994	Colorectal	+++	Liver	+++
JRR11842/11003	Colorectal	+	Liver	++
VFR13009/14142	Breast	+++	Bone	+
FFS4514/16987	Breast	++	Skin	+++
AUV4646/16954	Ovary	-	Skin	-
RMH18421/2795	Urinary bladder	++	Lung	++
CCR12186/8921	Melanoma	-	Bone	-
PLP9529/10689	Kidney	-	Skin	-

Quantitation of immunostaining in the tumours: (-): 0%; (+): 1-10%; (++): 11-50%; (+++): > 50%.

the vascular-endothelial tissue area was the positive control that serve us to quantified PECAM-1 expression in tumoral cells (**Figure 1**). Quantitation of immunostaining in the tumours was semiquantitatively assessed as (-): 0%; (+): 1-10%; (++): 11-50%; (+++): > 50%. The staining evaluation was assessed independently by two experimented pathologists.

3. Results

Immunohistochemical expression of PECAM-1 gave positive results in colon cancer, breast cancer and bladder cancer specimens (**Figure 2**). With the exception of bladder cancer the immunostaining for PECAM-1 was heterogeneous throughout the tumour sample with membranous and intracytoplasmic pattern.

As seen in **Table 1**, the immunostaining for PECAM-1 was positive in all colon, breast and urinary bladder cancers with different degrees of expression. In general, the expression was slightly more reduced in metastasic than in primaries tumours samples, with the exception of one of the colon cancer cases, which showed increased expression of the marker in the metastatic localisation (**Figure 3**). Immunohistochemical expression of PECAM-1 was not related to tumour grade or stage but interestingly in bladder cancer the expression of this protein was limited to the most basal layers.

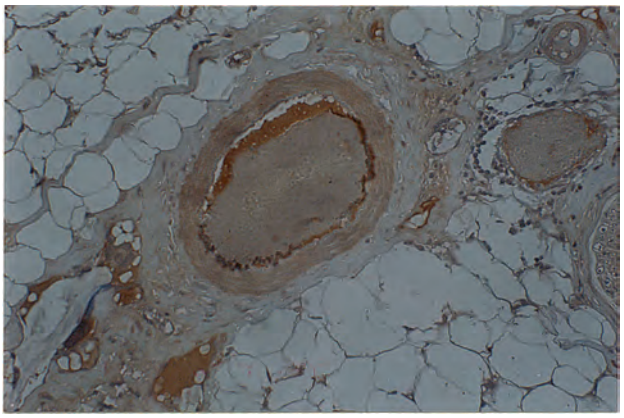

Figure 1. Tumoral Endothelial expression on PECAM-1

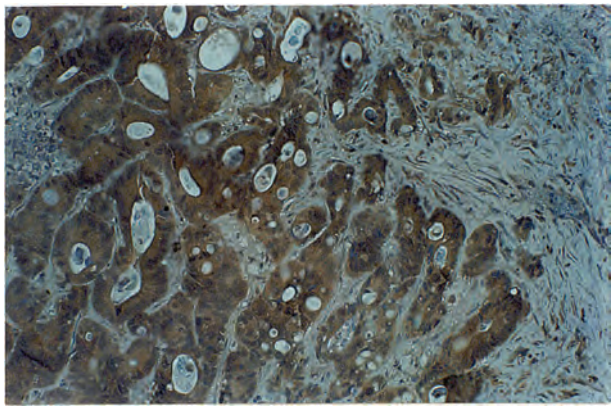

Figure 2. Expression of PECAM-1 in colorectal cancer

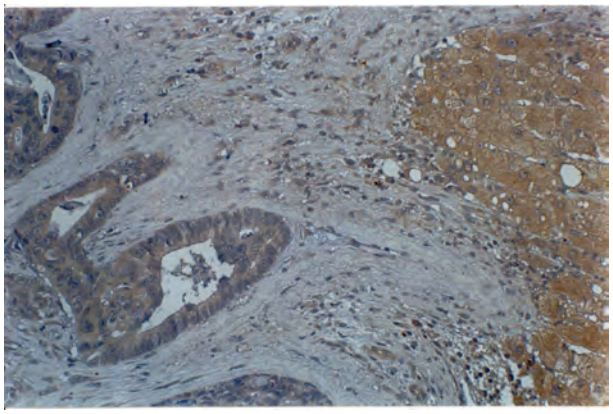

Figure 3. Expression of PECAM-1 in liver metastases of colorectal cancer

Melanoma and ovarian or kidney carcinomas did not show any immunohistochemical expression of PECAM-1 either in primary or metastatic sites.

4. Discussion

Although PECAM-1 expression has been studied in cellular lines of solid tumours, leukemias and lymphomas to the best of our knowledge the expression of this adhesion protein has not been evaluated in human tumours. Thus, the aim of the present study is to show this expression and the correlation between primary and metastatic localisation. This is important since it can strongly suggests that PECAM-1 may have a significant role in the metastatic process of some epithelial tumours, mainly colon cancer, breast cancer and probably bladder cancer and it could represent a different therapeutic approach by blocking PECAM-1 in some type of human cancers.

At the present we don't known whether the PECAM-1 expression in these tumours is constitutive or regulated by external stimuli. An interesting observation is the heterogeneous expression of PECAM-1 through the tumoral tissue in bladder samples. This can be explained by the heterogeneous character of neoplasms but it can also be argued that PECAM-1 expression identifies cellular clones which are more able to metastasize in epithelial tumours. Notwithstanding, PECAM-1 has been shown to be up-regulated by inflammatory cytokines (Rival, 1996; Romer, 1995) and the promoter region of this gene, which has been identified recently, contains sites responsive to transcription factors activated in the processes of cellular differentiation and proliferation (Almedro, 1996). Moreover, a functional site for the NF-kB factor has been also identified (Botella, 2000). It is well known that this transcription factor is stimulated by a wide range of external stimuli including mechanical factors and it is possible that cellular subgroups, located mainly at the basal cells layers, with higher proliferating activity express

PECAM-1 by means of the activation of transcription factor NF-kB. We are currently studying the possible correlation between PECAM-1 expression and NF-kB nuclear localisation in different areas of selected tumoral tissues.

Very little is known at present with respect to the function that PECAM-1 may have in tumour cells "in vivo", although some studies, as the one presented here, have suggested a possible role in metastatic spreading. Thus, it is possible that solid neoplasm expressing PECAM-1 are endowed with a greater capacity to migrate and disseminate. Nevertheless, we observed that some disseminated tumours do not show PECAM-1 expression and this result point out that the possible role of PECAM-1 in the tumoral cells is not universal, although the function of PECAM-1 in angiogenesis is well documented even in PECAM-1 negative tumours. Further studies are required to determine the important of PECAM-1 in the whole process of tumour progression as well as the molecular mechanisms that regulated the expression of this adhesion protein in the primary tumour.

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Analysis of Quality of Life in Cancer Patients by Structural Equation Model

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ABSTRACT

Many people have been dead of cancer. The life quality of patients with cancer has aroused great concern from the public and specialists. In this paper, an index system of life quality is proposed to evaluate the quality of life, which includes 6 *first-level indexes and* 34 *second-level indexes. Then, a structural equation model* (*SEM*) *based on these indexes and relationships among them is constructed for the analysis of quality of life in cancer patients. Furthermore, we offer a definite linear algorithm for the calculation of SEM. This method is more objective and scientific compared with traditional methods, such as descriptive analysis, some simple test methods and so on.*

Keywords*: *Quality of Life, Structural Equation Model, Unit Vector Constraint, Definite Linear Algorithm

1. Introduction

Nowadays, cancer has become the main threat to human health. For these diseases, it is hard to evaluate the treatment effect by cure rate, also survival rate is very limited. Compared with the traditional survival analysis method only considering survival rate or survival time, QOL evaluation focus more on subjective feeling, and can reflect the patient's health more fully. Considering that, the conception of QOL was accepted by the medical community in the 1970s，and there was ever a great upsurge in studies. QOL research of Clinical Oncology can go back to Karnofsky Performance Status (KPS) proposed by Karnofsky in the 1940s, which does not include the patient's subjective feelings, therefore, it is not a real QOL evaluation. The modern QOL research was initiated by Priestman and others with QOL measurement of breast cancer patients in 1976.QOL is an index system comprehensively evaluating individual physical, psychological, social support and demographic level.

QOL has been widely applied in various fields, and become an indispensable social important index and evaluation tool. The research of QOL has mainly three great effects in the treatment of cancer. 1) The evaluation of the effect of cancer treatment performs the selective therapy. 2) It has advantage of the selection and assessment of anti-cancer drug, antiemetic and anodyne and so on. U.S. FDA has decided that QOL must be the index in all anti-cancer drug evaluation, there should have the material both improving survival time and quality of life, when the efficacy of new medicine is not better than the old, new role can be found by QOL evaluation, which is good for drugs listed, it also helps to find new unexpected side effects or toxicity. 3) It helps to comprehend the long-term existing status of therapy in patients with cancer and assists to adopt the best management countermeasure in patients with late cancer. Now the basic principle of cancer radical treatment is the comprehensive treatment, the ultimate goal of which is to extend lifetime and improve the quality of life. But for most of the definite diagnosis of cancer patients, they already lost radical therapy and need palliative treatment, then improving QOL becomes the primary consideration. Therefore, study of QOL is of great significance in theoretically and practically, which needs further research.

At present, the evaluation of QOL is a very weak link, it is not maturity to measure QOL. Main systematic evaluation methods of Quality of Life, which are in common use are descriptive analysis method and some simple test methods, including Correlation Analysis and T test and so on. The assessment is seriously hindered by the complexity of QOL .In this paper, we build a related structural equation model (SEM) to reveal the potential factor of QOL, and the relationships between them, in which the summarizing coefficients are calculated by samples, so it is more objective and convincing, and could offer more deep analysis for the index systems. It will provide the suitable intervention methods in order to improve and enhance the quality of life in patients with cancer.

2. SEM for the QOL of the Cancer

SEM is a fast-growing embranchment of Application Statistics, widely used in psychology, sociology and other fields. This model not only studies the interior relationship among various factors, but also the relative and causal relations among latent variables. The QOL of the patients is controlled by psychological factor and physiological factor, whose values cannot be measured directly, so it is not suitable to use the usual statistical methods to analyze the influencing factors of the QOL. Considering that, this paper is the application of SEM to analyze the influencing factors of the QOL in patients with cancer.

There are always two systems of equations in a SEM. One is a structure system of equations among structural variables, and the other one is a measurement system of equations between structural variables and observed variables. Since the QOL of the patients with cancer cannot only reflect the curative effect, but also prevention and rehabilitation effects, it's important and urgent to quantitative analysis and build effective QOL index immediately. In our model, there are 6 latent variables and 34 observed variables. The variables are listed in **Table 1** as follows:

There are 6 structural variables ($\xi_1, \eta_1 \sim \eta_5$) and 15 path relationships, which are expressed in **Figure 1** below (The path coefficients from the exogenous latent variable ξ_1 to the endogenous latent variables $\eta_1 \sim \eta_5$ are $\gamma_1 \sim \gamma_5$, expressed with dashed arrowheads; The relationships among independent variables are β_{ij}, expressed with real-line arrowheads).

The structural Equations are relationships among the latent variables. The structural equations can be expressed as follows:

$$\begin{pmatrix}\eta_1\\ \eta_2\\ \eta_3\\ \eta_4\\ \eta_5\end{pmatrix}=\begin{pmatrix}0 & 0 & 0 & 0 & 0\\ \beta_{21} & 0 & 0 & 0 & 0\\ \beta_{31} & \beta_{32} & 0 & 0 & 0\\ \beta_{41} & \beta_{42} & \beta_{43} & 0 & 0\\ \beta_{51} & \beta_{52} & \beta_{53} & \beta_{54} & 0\end{pmatrix}\begin{pmatrix}\eta_1\\ \eta_2\\ \eta_3\\ \eta_4\\ \eta_5\end{pmatrix}+\begin{pmatrix}\gamma_{11}\\ \gamma_{12}\\ \gamma_{13}\\ \gamma_{14}\\ \gamma_{15}\end{pmatrix}\xi_1+\begin{pmatrix}\varepsilon_1\\ \varepsilon_2\\ \varepsilon_3\\ \varepsilon_4\\ \varepsilon_5\end{pmatrix} \tag{1}$$

In general, vector and matrix are used to describe the structural Equations. Let $\xi' = (\xi_1', \cdots, \xi_k')$ and $\eta' = (\eta_1', \cdots, \eta_m')$. Then $m \times m$ square matrix B is the coefficient matrix of η, then $m \times k$ matrix Γ is the coefficient matrix of ξ, $\varepsilon_\eta' = (\varepsilon_1', \cdots, \varepsilon_m')$ is the residual vector, then the structural Equations (1) may be extended as:

$$\eta = B\eta + \Gamma\xi + \varepsilon_\eta \tag{2}$$

The structural variables are implicit and cannot be observed directly. Each structural variable is corresponding with many observed variables.

Suppose there are k independent structural variables and m dependent structural variables. The observed variables corresponding to the independent structural variable ξ_t are denoted as x_{tj}, $t = 1, \cdots, k$, $j = 1, \cdots, K(t)$, where $K(t)$ is the number of observed variables corresponding to the independent structural variable ξ_t. In **Figure 1**, $k = 1$ and $K(1) = 6$. The observed variables corresponding to the dependent variable η_i are denoted as y_{ij}, $i = 1, \cdots, m$, $j = 1, \cdots, L(i)$, where $L(i)$ is the number of observed variables corresponding to the dependent structural variable η_i. In **Figure 1**, $m = 5$ and $L(i) = 6, 6, 6, 4, 6$.

The observation equations can be expressed as the relationship from the observed variables to the structural variables:

$$\xi_t = \sum_{j=1}^{K(t)} \psi_{tj} x_{tj} + \varepsilon_{xt}, \quad t = 1, \cdots, k \tag{3}$$

$$\eta_i = \sum_{j=1}^{L(i)} \omega_{ij} y_{ij} + \varepsilon_{yi}, \quad i = 1, \cdots, m \tag{4}$$

where ψ_{tj}、ω_{ij} are the summarizing coefficients, and ε with subscript is a random error.

The relationships between the structural variables and the observed variables can also be expressed as follows:

$$\begin{pmatrix}x_{t1}\\ \vdots\\ x_{tK(t)}\end{pmatrix}=\begin{pmatrix}\upsilon_{t1}\\ \vdots\\ \upsilon_{tK(t)}\end{pmatrix}\xi_1+\begin{pmatrix}\varepsilon_{xt1}\\ \vdots\\ \varepsilon_{xtK(t)}\end{pmatrix}, \quad t = 1, \cdots, k \tag{5}$$

$$\begin{pmatrix}y_{i1}\\ \vdots\\ y_{iL(i)}\end{pmatrix}=\begin{pmatrix}\lambda_{i1}\\ \vdots\\ \lambda_{iL(i)}\end{pmatrix}\eta_i+\begin{pmatrix}\varepsilon_{yi1}\\ \vdots\\ \varepsilon_{yiL(i)}\end{pmatrix}, \quad i = 1, \cdots, m \tag{6}$$

where υ_{tj}、λ_{ij} are the carrying coefficients, and ε with subscript is still a random error.

Denoting observation vectors as $x_t' = (x_{t1}', \cdots, x_{tK(t)}')$, $y_i' = (y_{i1}', \cdots, y_{iL(i)}')$, and denoting coefficients as

Table 1. Index of variables

Structural variables	Observed variables		
Demographic factors ξ_1	Age x_{11}	Sex x_{12}	Character x_{13}
	Level of education x_{14}	Occupation x_{15}	Marital status x_{16}
Social support η_1	Interpersonal relationship y_{11}	Family ties y_{12}	cognitive structure y_{13}
	Nursing y_{14}	Economic capability y_{15}	Medical facilities and service y_{16}
Types of cancer η_2	Cancer sites y_{21}	Cancer incidence rate y_{22}	Cure rate y_{23}
	Cancer benign or malignant y_{24}	Cancer period y_{25}	Type of medical care system y_{26}
Physiological factors η_3	The degree of physical pain y_{31}	Fatigue y_{32}	Anorexia y_{33}
	Health condition before the cancer y_{34}	Natural immunity y_{35}	Nutritional state y_{36}
Psychological factors η_4	Personal knowledge of cancer y_{41}	Self assessment y_{42}	Emotional stability y_{43}
	Psychological therapy y_{44}		
The quality of life η_5	Emotions and psychology y_{51}	Functional status y_{52}	Satisfaction rate with the treatment y_{53}
	Hope for the future y_{54}	Survival time y_{55}	Life satisfaction y_{56}

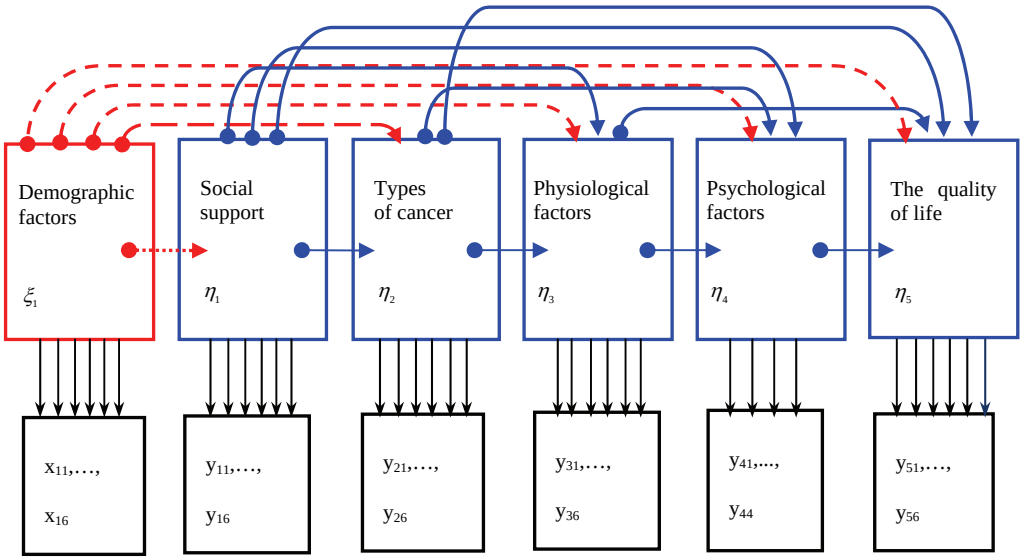

Figure 1. Model of QOL index

$\psi_t' = (\psi_{t1}', \cdots, \psi_{tK(t)}')$, $\omega_i' = (\omega_{i1}', \cdots, \omega_{iL(i)}')$, then the Equations (2)-(4) can be written into:

$$SEM^+ \begin{cases} \eta = B\eta + \Gamma\xi + \varepsilon_\eta \\ \xi_t = \psi_t' x_t + \varepsilon_{xt}, t = 1, \cdots, k \\ \eta_i = \omega_i' y_i + \varepsilon_{yi}, i = 1, \cdots, m \end{cases} \quad (7)$$

We call SEM^+ the structural equation model with positive observation.

Letting $\upsilon_t' = (\upsilon_{t1}', \cdots, \upsilon_{tK(t)}')$, $\lambda_i' = (\lambda_{i1}', \cdots, \lambda_{iL(i)}')$, then the observation Equations (5) and (6) can be expressed as:

$$x_t = \upsilon_t \xi_t + \varepsilon_{xt}, \quad t = 1, \cdots, k \quad (8)$$

$$y_i = \lambda_i \eta_i + \varepsilon_{yi}, \quad i = 1, \cdots, m \quad (9)$$

Combine the Equations (2), (8) and (9) as:

$$SEM^{-}\begin{cases}\eta = B\eta + \Gamma\xi + \varepsilon_{\eta}\\ x_t = \upsilon_t \xi_t + \varepsilon_{xt},\ t = 1,\cdots,k\\ y_i = \lambda_i \eta_i + \varepsilon_{yi},\ i = 1,\cdots,m\end{cases} \tag{10}$$

and call SEM^{-} the structural equation model with converse observation.

3. The Least Square Solution by the Modular Constraint of Structural Vector

By analyzing the observation equations of SEM carefully, we can discover the least square relationship between each structural variable and its observed variables, and obtain the least square solution of structural variable by the modular constraint of structural vector. Now, we give the MCLS algorithm of structural vector in SEM which is studied by penman as follows:

Algorithm 1. The modular constraint least square solution (MCLS) of structural vector in SEM.

Step 1. In SEM^{-}, suppose that ξ_t, η_i all are unit vectors, calculate the least square estimates of the coefficients between the structural variable and its observed variables:

$$\hat{\upsilon}_{tj}^{2} = x_{tj}x'_{tj},\quad j = 1,\cdots,K(t),\quad t = 1,\cdots,k \tag{11}$$

$$\hat{\lambda}_{ij}^{2} = y_{ij}y'_{ij},\quad j = 1,\cdots,L(i),\quad i = 1,\cdots,m \tag{12}$$

Step 2. In SEM^{-}, calculate the least square estimates of structural variable by making use of $\hat{\upsilon}_{ij}, \hat{\lambda}_{ij}$:

$$\hat{\xi}_{ts} = \frac{\hat{\upsilon}_t' X_{ts}}{\hat{\upsilon}_t \hat{\upsilon}_t'},\quad \hat{\eta}_{is} = \frac{\hat{\lambda}_i' Y_{is}}{\hat{\lambda}_i \hat{\lambda}_i'}, s = 1,\cdots,N,$$
$$t = 1,\cdots,k,\quad i = 1,\cdots,m \tag{13}$$

Here X_{ts}, Y_{is} are the transverse vector of the observation data matrix, $X'_{ts} = (x_{t1s},\cdots,x_{tK(t)s})$, $Y'_{is} = (x_{i1s},\cdots,x_{iL(i)s})$.

Step 3. In SEM^{+} (or (3) and (4)), make use of $\hat{\xi}_t, \hat{\eta}_i$ obtained in Step 2 to calculate regression coefficients ψ_{tj}, ω_{ij} according to a common linear regression method.

Step 4. In SEM^{+} (or (2)), make use of $\hat{\xi}_t, \hat{\eta}_i$ obtained in Step 2 to calculate the estimates of coefficient matrices B, Γ.

Notice that (2) is a common linear regression Equation system, we can use Two Step Least Square to calculate it.

4. Definite Linear Algorithm of Prescription Constraint

Since the solutions of SEM^{+} or SEM^{-} are not unique, and they may differ by a multiple (the proof is omitted). Therefore, in the structural Equation (1) or (2), if each structural variable is multiplied by the same multiple, its coefficient solution is the same. Taking note of this, the solution of structural equations is irrelevant to the modular length of the structural variable. However, it is not reasonable to assume that the modular length of each structural variable is 1. On the other hand, if each modular length of the structural variable is not the same in the possibly existing optimal solution set, then MCLS is not good.

One reasonable way is to let each structural variable have an undetermined parameter of the modular length and combine the structural Equation (1) or (2) to find the solution. The error square sum of this solution includes $m+k$ modular length parameters. Changing these modular length parameters to minimize the error square sum, we can obtain a reasonable modular length of the structural variable.

Another possible way is to find a more reasonable constraint to replace the modular constraint. After getting MCLS, we can change the modular length of the structural variable in observation equations to make the path coefficient between structural variables and observation variables satisfy the prescription condition. In Equations (3) and (4), the prescription conditions are:

$$\sum_{j=1}^{K(t)} \psi_{tj} = 1,\quad \psi_{tj} \geq 0,\quad t = 1,\cdots,k \tag{14}$$

$$\sum_{j=1}^{L(i)} \omega_{ij} = 1,\quad \omega_{ij} \geq 0,\quad i = 1,\cdots,m \tag{15}$$

If the corresponding path coefficients of MCLS are non-negative at the beginning, then it is simple. We just need to divide the two sides of the Equations (3) and (4) by a constant. This constant should be the sum of the corresponding path coefficients in MCLS. For example, in the Equation (3), if $\sum_{j=1}^{K(i)} \psi_{ij} = c_i$, then the two sides of the Equation (4) are divided by the constant c_i, so the modular length of structural variables becomes $1/c_i$, and $\sum_{j=1}^{K(i)} \psi_{ij} = 1$.

If the corresponding path coefficients of MCLS are negative at the beginning, we cannot copy the method of prescription regression proposed by Fang (1982, 1985), because regression dependent variables are not completely known. Now we know the direction of regression dependent variables, but the modular length is undetermined. According to the theorem in Fang (1982), if the initial regression coefficients have negative ones, whose

prescription regression coefficient should be 0. So we can first make ordinary regression about MCLS, where the modular length of dependent variables is 1. If there are some non-positive terms in the initial regression coefficients, we can get rid of these variables, and thus the corresponding regression coefficient is 0. Then the two sides of the Equations (3) and (4) can be divided by a constant that should be the sum of the corresponding path coefficients in MCLS, as discussed in the previous paragraph.

Of course we can improve the constraint of the prescription condition. If some regression coefficient is 0, its corresponding variable may be removed from the model, which is not a desired situation. To avoid this, we may change the prescription condition and let $\psi_{tj} \geq \delta$, $\omega_{ij} \geq \delta$, where $\delta > 0$. If some initial regression coefficients are less than δ, they all are changed as δ, and the corresponding independent variables with coefficient δ should be moved to the left side of the equation in regression process.

Summarizing the above discussion we can continue to improve the algorithm of MCLS.

Algorithm 2. Improvement on Step 3 of Algorithm 1.

Step 5. After getting the estimate of structural variables $\hat{\xi}_t, \hat{\eta}_i$ in Step 2, calculate the summarizing coefficients ψ_{tj}, ω_{ij} by prescription regression, and recalculate the estimates of ξ_t, η_i.

1) Make use of $\hat{\xi}_t, \hat{\eta}_i$ directly in Step 2 and calculate $\hat{\psi}_{tj}, \hat{\omega}_{ij}$ in SEM^+ by common regression.

2) For any t, if there are $\hat{\psi}_{tj} \geq \delta$, ($\delta \geq 0$) for all j, and $\sum_{j=1}^{K(t)} \psi_{tj} = c_t$, then divide both sides of Equation (3) by c_t. Similarly, for any i, if there are $\omega_{ij} \geq \delta$, ($\delta \geq 0$) for all j, and $\sum_{j=1}^{L(i)} \omega_{ij} = c_i$, then divide both sides of Equation (4) by c_i.

After checking all t, i, go to Step 4 in Algorithm 1.

3) For any t, i, if there is some j so that $\hat{\psi}_{tj} < \delta$, or $\omega_{ij} < \delta$, ($\delta \geq 0$), then let the corresponding term be fixed, *i.e.*, $\hat{\psi}_{tj} = \delta$ or $\omega_{ij} = \delta$. After checking all j, go to Step 1 and Step 2 in this algorithm.

Note that if some regression coefficient is fixed in common regression, the corresponding independent variables with its coefficient δ should be moved to the left side of the equation and combined with the dependent variable to regression. After regression the corresponding independent variable with its coefficient δ should be moved to the right side of the equation.

5. Conclusions and Discussions

In this paper, a structural equation model is proposed to analyze the quality of life in cancer patients. Besides, a definite linear algorithm for SEM based on the modular constraint of structural variable, the least square theorem, and the prescription regression method is introduced, this model is more objective and scientific compared with traditional methods, such as descriptive analysis method and some simple test methods, including Correlation Analysis and T test and so on, because the summarizing coefficients of this evaluation system are calculated by samples rather than designed arbitrarily. Therefore, we can have a better understanding of the relationships among the indexes, which will do a great favor to decision-making analysis of Quality of Life in Cancer Patients.

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Mechanisms and Immune Dysregulation in Arsenic Skin Carcinogenesis

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ABSTRACT

Long-term exposure to arsenic is associated with cancers of lung, urinary bladder, kidney, liver and skin. Arsenic carcinogenesis might result from oxidative stress, altered growth factors, chromosomal abnormality, immune dysregulation, and aberrant epigenetic regulations. Bowen's disease (*As-BD*) *is the most common form of arsenic-induces skin cancers and is characterized by chronicity, multiplicity, and predisposition in sun-spare skin. However, only about* 1% *of the population exposed to arsenic developped skin cancers, indicating the host immune response plays an important modulatory role in skin carcinogenesis. In this review, we review the pathomechanisms of arsenic skin carcinogenesis and the immune interactions. Arsenic affects innate and adaptive immune responses through CD*4+ *T cells, monocytes, macrophages, and Langerhans cells. In skin of As-BD, CD*4+ *T cells undergo selective and differential apoptosis via Fas-FasL interaction. Numbers and dendrites of Langerhans cells are reduced in As-BD lesions. There is a defective homeostasis and aberrant trafficking of Langerhans cells. Such information is essential to understand the molecular mechanism for arsenic carcinogenesis in both skin and in internal organs.*

Keywords**:** *Arsenic, Bowen's Disease, Skin Cancer, Innate Immunity, Langerhans Cells*

1. Introduction

Arsenic is a ubiquitous element on the earth. People may expose to arsenic in several ways through drinking, inhalation, and direct skin contact. Drinking of water with arsenic remains the major route of human exposure [1], leading to development of cancers of skin, lungs, and liver in many countries, including Bangladesh, Taiwan, West Bengal of India, Chile, Mexico, and China [2]. Industrially, arsenic is used to generate paints and insecticides. Arsenide is also a critical constituent in semiconductors that is used for electronic chips and computers. In addition to its carcinogenic property, arsenic exposure is also associated with vascular diseases, including stroke, ischemic heart diseases, and peripheral vascular disease [3]. In contrast to its notoriously adverse health effects, arsenic has been used for treatment of lymphoma and leukemia and it still remains the drug of choice for acute promyelocytic leukemia.

It is estimated that around 10% of population exposed to arsenic will develop skin abnormalities, including variegated hyper-/hypo-pigmentations, arsenic keratosis, Bowen's disease, and invasive skin cancers. Only about 1% of exposed population develops skin cancers. Long-term arsenic exposure results in impaired immunity in susceptible individuals, which may account for the development of cancers in vulnerable individuals. Personal genetic variability, immune system, and the interaction of both might differ, leading to differential susceptibility and cancer immunity in the process of arsenic carcinogenesis.

Based on its oxidative status, arsenic exists in two inorganic chemical forms. Arsenite (AsIII) is a trivalent form while arsenate (AsV) is a pentavalent form. Trivalent arsenite is about 2-10 times more toxic than pentavalent arsenate. In tissues, arsenic is methylated by methyl group supplied by s-adenosylmethionine (SAM). Compared to inorganic forms, the methylated metabolites are less genotoxic [4] and are excreted more quickly in urine [5,6]. After ingestion, inorganic arsenic is obtained by erythrocytes and then distributed systemically to multiple organs, including lungs, liver, and skin [7,8]. In transit from blood to tissues, arsenate is reduced to arsenite. In the liver, arsenic is methylated into monomethylarsenic acid (MMA V), which can be further reduced to monomethyl arsonous acid (MMA III). A further methylation reaction modifies MMA III to dimethylarsinic acid (DMA V) [9]. In the process of methylation

process, several reactive oxygen species (ROS) are generated [10,11]. WHO recommends safe groundwater arsenic concentration up to 50 μg/L but proposes a provisional arsenic standard at 10 μg/L [12]. The government regulation for arsenic contents in drinking water depends on countries but the standard generally is more stringent, for example, up to 10 g/L in Taiwan, Japan, and U.S.

One of the most systematized epidemiological studies for health effects of arsenic is conducted in Taiwan and it has led the basis of many epidemiological risk assessments over the last 40 years worldwide [6]. In the past decades, we have been investigating on the arsenic carcinogenesis with particular focus on skin not only it is readily accessible but also it might provide a model of chemical carcinogenesis and immune interaction. This review discusses the pathomechanisms of arsenic skin carcinogenesis with special focus on the interactions of immune system and arsenic-induced cancers.

2. Proposed Mechanisms of Action in Arsenical Carcinogenesis

Although arsenic is documented as a weak mutagen, the International Agency for Research on Cancer (IARC) has categorized arsenic as a human carcinogen [13]. The mechanism of arsenic carcinogenesis remains uncertain. However, oxidative stress, chromosomal abnormality and altered growth factors may contribute to arsenic carcinogenesis [9,14,15]. It has been suggested that arsenic might act as a co-carcinogen or a promoter in carcinogenesis by mode of action studies [16]. However, recent studies showed perinatal maternal exposure to arsenic results in spontaneous cancer developments in off springs, suggesting that arsenic might also act in the initiation in two stage chemical carcinogenesis [17]. The effects of arsenic exposure in early life development include epigenetic effects, via DNA hypomethylation, endocrine effects (most classes of steroid hormones), immune suppression, neurotoxicity, and interaction with enzymes critical for fetal development and programming [18].

Arsenic tends to bind to the thio-group (-SH) of proteins, targeting regulatory or structural proteins [19,20]. Approximately 200 proteins could be targeted by the bindings and interactions of arsenic-thio group [21]. Among these proteins, the proto-oncogene c-Jun is well investigated. By binding to thio-groups, arsenic can block Jun N-terminal kinase (JNK) phosphatase activity, resulting in an over activation of JNK, which activates proto-oncogene c-Jun, inducing c-Jun/c-Fos (AP-1)-mediated gene upregulations [22-24]. These upregulated genes include cell cycle regulation, and apoptotic signaling, all of which are strongly linked to arsenic carcinogenesis. Moreover, we have shown there is a quantitative impairment of phosphorylation of keratin 1 and keratin 2 in the process of chronic arsenism, suggesting that keratins, containing plenty of thio groups, are the cellular targets of arsenic [25]. Through oxidative stress induced by arsenic, genomic mutations might develop, leading to initiation in carcinogenesis. Several lines of compelling evidences revealed the oxidative DNA damages in the target organ of arsenic-exposed animals and humans. In fact, clinical studies in arsenic-induced Bowen's disease (As-BD) uncovered the correlations between increased 8-OHdG levels and the arsenic concentration in the lesional skin [26], indicating the importance of oxidative stress in arsenical skin carcinogenesis. Mechanistically, in vitro studies showed low concentrations of arsenic (< 5 μM) can generate ROS, which in term increases the transcription of the nuclear factor kappa B (NF-KB) [24,27-30], that eventually promotes cell proliferation [31].

The second possible mechanism in arsenic carcinogenesis is through genomic instability and chromosome abnormalities. Arsenic is repeatedly reported to induce chromosome abnormalities and aberrant sister chromatid exchanges [32-34]. In human fibroblasts and CHO cells, arsenic induced chromosome abnormalities and induces sister chromatid exchanges at high and low concentrations, respectively [35,36]. These chromosomal abnormalities were highly associated with arsenic-induced oxidative DNA damages [26,37] and might link to arsenic carcinogenesis. Arsenite exposure induced micronuclei (MN) formation [38], which indicates cellular response to DNA damages. An increased frequency of MN was also detected in exfoliated bladder cells, buccal cells, and lymphocytes from arsenic-exposed humans [39-41]. Chien et al showed that arsenite results in tumorigenicity of HaCaT cells in nude mice by increased frequency of MN [42].

The third possible etiological factor leading to arsenic carcinogenesis is through abnormal DNA repair and epigentic regulations. Arsenic was able to inhibit DNA repair systems in the steps of nucleotide excision repair [43-45], DNA ligase III activity, DNA base excision repair [46,47] and DNA strand break rejoining [48]. Many key DNA repair regulatory proteins were inhibited by arsenic, including DNA ligase I, DNA ligase II, DNA ligase III, DNA polymerase β, 6-methyl-guanine-DNA methyltransferase, and poly (ADP-ribose) polymerase (PARP) [14,47,49]. Agents messing up with those DNA repair proteins can lead to genetic mutations. Along with its effect in DNA repair, arsenic also potentiated the mutagenicity of other carcinogeneic factors (such as UV, X-rays, and chemical agent) [50-53]. Moreover, arsenic affected global histone methylation and also DNA methylation, indicating that arsenic also affects epigenome machinery to influence gene expressions involved in carcinogenesis [54]. In most cases, arsenic induced DNA hypomethylation, probably through the inhibition of DNA methyltransferases [55]. However, it has been reported there is a hypermethylation in promoter of gene

p53 and p16 in people exposed to arsenic [56]. Arsenic at very low concentrations (below 1μM) can inhibit both DNMT1 and DNMT3A in HaCaT cells [57]. Indeed, recent studies showed perinatal exposure to arsenic results in DNA methylation globally in GC-rich (guanidine and cysteine) regions [58].

3. Hophysiology of Arsenic Skin Cancers

Arsenic tends to accumulate in ectodermal tissues including the skin, hair and nails. Skin lesions are most common and most accessible in arsenic-induced pathologies [59-61]. Variegated hyper- and hypo-pigmentation and punctate palmar-plantar hyperkeratosis are all hallmarks of chronic arsenic exposure. The hyperkeratosis may appear as a regular thickening or as discrete nodules. A dose relationship has been found for the arsenic concentrations in well water and the occurrences of hyperpigmentation and hyperkeratosis among the people living in the endemic areas [62,63]. Furthermore, Tseng *et al.* found a dose-response relationship between arsenic levels in drinking water and skin cancers by a comprehensive epidemiological survey five decades ago [62]. Among skin cancers, the most common arsenic-induced skin cancers are Bowen's disease, followed by basal cell carcinoma and squamous cell carcinoma [62].

Bowen's disease is a carcinoma in situ of the skin resulting from UV or arsenical exposure [2,59,62,64]. Clinically, arsenic-induced Bowen's disease is different from classical (UV-induced) Bowen's disease by the its multiplicity and its propensity in sun-spare skins [2,62,65]. There are abnormal cellular proliferation and apoptosis in arsenic-induced Bowen's disease (As-BD) as presented with increased epidermal thickness and individual dyskeratotic keratinocytes, respectively [2,59]. After decades of development, As-BD can penetrate through basement membrane and become invasive squamous cell carcinoma (SCC), basal cell carcinoma (BCC), and combined forms of the skin cancer [2,62, 66,67]. Individuals with As-BD are considered a risk for development of malignancies in the lung and urinary bladder [67-70]. It was estimated that As-BD started within one decade, invasive skin cancer after scores of years [71], and lung cancers after 30 years following the chronic arsenic exposure [66]. Therefore, the characteristic clinical and histopathological features of As-BD serve as a model to understand the different stages of chemical carcinogenesis.

Microscopically, p53 protein was greatly expressed in As-BD as compared with non-arsenical BD [72-74]. Arsenic can induce p53 accumulation via an ATM-dependent pathway [75,76]. The over-expressed p53 in As-BD lesions was a mutant form [77,78], of which most of the p53 mutation sites are located on exon 5 and exon 8. Furthermore, the mutation types of p53 gene mutation in As-BD were different from those in UV-related skin cancers [79], indicating the differences in the pathogenesis of As-BD and UV-induced Bowen's disease. Although the connection between p53 mutation and arsenic exposure was not clear, the effect of arsenic on p53-related pathways was well recognized. Studies have shown that arsenic exposure resulted in G2/M cell cycle arrest and DNA aneuploidy, both of which are regulated by p53 [80-82].

There are coexisting hyperproliferative and dyskeratotic (apoptotic) keratinocytes in As-BD lesions [31]. The effects of arsenic on keratinocytes depend on the concentrations of arsenic. At lower concentrations (≦ 1 μM), arsenic induced keratinocyte proliferation and enhanced both NF-KB and AP-1 activity [31]. The proliferation is dependent on the mitochondrial biogenesis (manuscript in preparation). At higher concentrations (≧ 5 μM), arsenic induced keratinocyte apoptosis by Fas/Fas ligand (FasL) pathway. Since promoter regions of FasL contained binding sites for AP-1, arsenic-activated Fas/ FasL signaling may associate with arsenic-induced AP-1 activation [83-85].

4. Arsenic Influences Immune Regulation and Immune Responses

Intact and functional immunity is important in tumor surveillance of skin cancers. This is evidenced by the fact that patients with renal transplant and HIV infection have higher risk to develop skin cancers. Depends on cell type, tissue, and species, arsenic influenced immune system and its responses differentially in many aspects [86-88]. Arsenic may provoke immune responses by inhibition of regulatory or suppressor cells. For example, arsenic affected function of regulatory T lymphocytes in humans [89]. Arsenic in vitro enhanced immune response by deleting the precursors of suppressor T cells from normal spleen cells [90].

Arsenic affected many different kinds of cells in the immune system, leading to dysregulated immune responses. In utero exposure to arsenic impaired child thymic development and enhanced morbidity to respiratory infection [91]. The increase in respiratory infection by influenza was also shown in arsenic-exposed mice [92] and was associated with alternation of immune response genes in the lungs, such as IL-1beta, IL-1R and a number of toll-like receptors [93]. In addition to its adverse effects on the infection, arsenic can alter the systemic immunity. In patients with As-BD, there was a markedly reduced in contact hypersensitivity (CHS) reaction to DNCB in the skin [94], accompanied with reduced spontaneous and phytohemaglutinin(PHA)-induced IFN-gamma and TNF-alpha productions. The decreased CHS response to DNFB was also shown in the mice fed with arsenic-containing water [95]. We reported that arsenic induces CD4+ cells apoptosis by affecting the autocrine TNF-alpha loop [96,97]. Furthermore, there was a de-

crease in numbers of T cells and the expression of IL-2R on them from patients with As-BD [94,98]. We also showed that T cells from arsenic-exposed people were anergy to PHA stimulation but were exaggerative to arsenic treatment [96]. A recent study showed T cell proliferation to Concanavalin A (Con A) was markedly reduced in people exposed to arsenic and there was a parallel decrease in the levels of TNF-alpha, IFN-gamma, IL-2, IL-10, IL-5, and IL-4 [99]. In arsenic-exposed children, arsenic burden was also associated with a reduced proliferative response to PHA stimulation. CD4+ cells were selectively decreased with CD8+, B, and NK cells remained unaffected in proportion. IL-2 but not IL-4, IL-10, or IFN-gamma was decreased in PHA-activated peripheral blood mononuclear cells [100]. In mice, T cell-dependent humoral immune response was extremely sensitive to suppression by arsenic and assessment of humoral immune responses should be considered in evaluating the health effects of arsenic containing agents [101]. Collectively, arsenic inhibits systemic T cell activation and proliferation via TNF-alpha axis. Macrophages were also potential targets of arsenic in humans [102]. Macrophages from people exposed to arsenic showed loss of cell adhesion capacity, decreased in NO production, and impaired phagocytic ability [103]. Monocytes from children exposed to arsenic produced less superoxide anion and nitric oxide[104]. Arsenic also affected phagocytic ability of neutrophils and degranulation via Syk activation[105].

In the cell level, arsenic has dual effects on cell proliferation in lymphocytes. Arsenic compounds at low concentrations enhanced DNA synthesis in PHA-stimulated proliferation of human lymphocytes, whereas arsenic at higher concentrations inhibited cellular proliferation and induced apoptosis [96,106,107]. As occurred in fibroblasts and CHO cells, arsenic has been shown to induce aneuploidy [108,109], sister chromatid changes [110-112], other chromosomal abnormalities [32] in lymphocytes. Mechanistically, arsenic influenced T cell receptor activation by increasing basal and induced phosphorylation of Lck and Fyn (first kinase associated to TCR complex) [113]. Metabolites of arsenic can also interfere with cell division via tubulin disruption, leading to aneuploidy [114]. A major cellular source of methyl group, S-adenosyl-L-methionine can reverse micronucleus formation induced by sodium arsenite and other cytoskeleton disrupting agents in cultured human lymphocytes [115].

In contrast to the scarce studies studying effects of arsenic on normal lymphocytes, there are a lot of studies investigating arsenic induced apoptosis in cells from lymphoma or leukemia, which coincides with the use of arsenic trioxide in acute promyelocytic anemia and multiple myeloma. Arsenic induced normalization of differentiation of promyelocytes in acute promyelocytic leukemia, of which arsenic remains the drug of choice. Phosphoinositide 3-kinase/Akt inhibition increased arsenic trioxide-induced apoptosis of acute promyelocytic and T-cell leukemia [116]. Arsenic induced apoptosis through activation of Bax in hematopoietic cells [117]. Furthermore, arsenic trioxide (As) and interferon (IFN)-alpha coordinately induced cell cycle arrest and apoptosis that is modulated by bcl-2, bax, p53, and NF-kappaB [118,119]. Thus, the effects of arsenic on T cells development may act as a double-sided sword and appear to be cell-specific and concentration-dependent.

5. Altered Skin Associated Lymphoid Tissue in Arsenic-Induced Bowen's Disease

In patients with As-BD, there was a markedly reduced in contact hypersensitivity reaction to DNCB in the skin [94], accompanied with reduced spontaneous and PHA-induced IFN-gamma as well as TNF-alpha production. Both GM-CSF and TGF-alpha were found in the epidermis at clinically normal sites within 10 weeks after arsenic treatment in vivo and also from arsenic-treated keratinocytes [120-121]. Arsenic can enhance keratinocytes to express TGF-α, GM-CSF, IL-6 and IL-8 [120-122]. These growth factors and cytokines expression may induce cutaneous tumorigenesis via AP-1 and NF-KB regulation [123]. Although a clear link has been established for impaired T cell proliferation by arsenic, the causative role of impaired T cell activation and predilection of T cell apoptosis with the cutaneous carcinogenesis has seldom discussed. There was a decreased proportion of peripheral CD4+ cells in the peripheral blood from arsenic-exposed humans as compared to that from control subjects; the CD4+ cells from As-BD patients were less vulnerable to arsenic-induced apoptosis, due to defected TNF-R1 expression. Those residual CD_4^+ cells were less susceptible to arsenic-induced apoptosis. However, when those CD4+ cells infiltrated into the As-BD lesions, FasL from As-BD in the epidermis induced selective CD4+ cell apoptosis (**Figure 1**). This additional tumor evasion phenomenon present in the cutaneous environment provided a reasonable explanation for persisting nature of arsenic cancers in the skin despite the moderate dermal inflammatory infiltrates [124].

It is evident that cell-mediated immunity is depressed in patients with arsenic-induced Bowen's disease [94]. Langerhans cells (LC) are known to be one of the professional antigen presenting cells for T lymphocytes. They play a pivotal role in the presentation of tumor-associated antigens. Others and we have reported there was a progressive decrease in numbers of Langerhans cell in the order of normal skin, normal appearing skin in As-BD, and As-BD lesion. [125-127]. However, how arsenic alters LC migration and polarizes Th responses remains unknown. Using an epicutaneous protein sensitization model in mice, we have found that arsenic exposure enhanced LC migration to draining lymph nodes, and

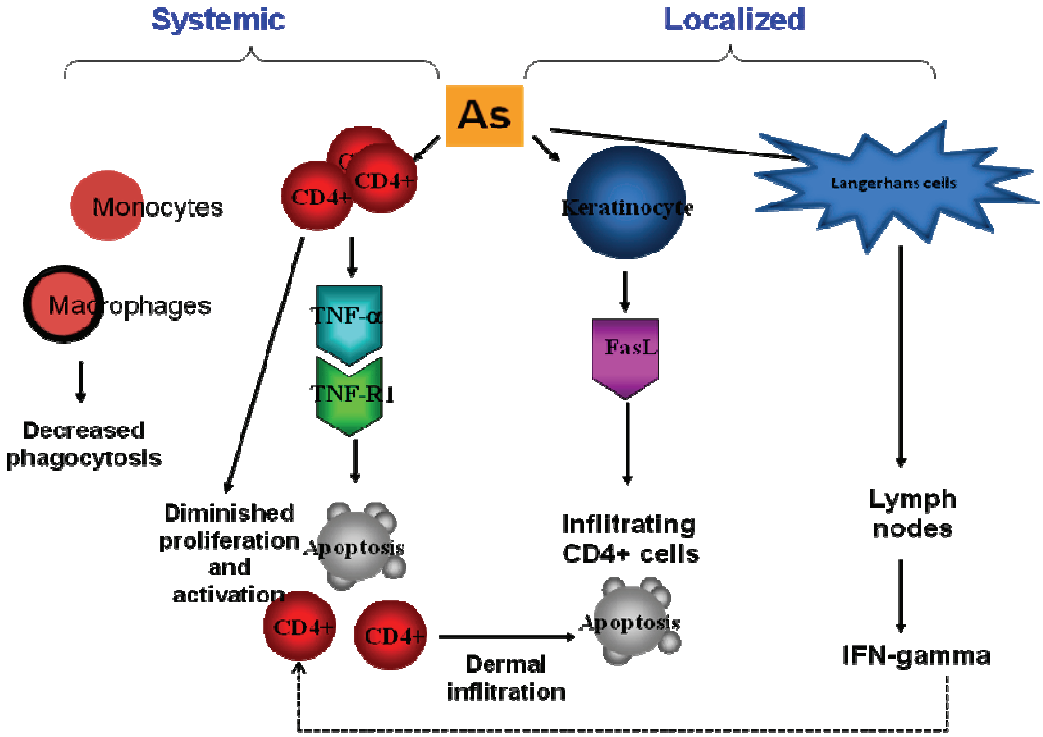

Figure 1. Proposed altered systemic and cutaneous immune responses in arsenic carcinogenesis. Arsenic inhibits T cell proliferation, induces T cell apoptosis, decreases monocyte/macrophage phagocytosis in the systemic circulation. In the skin, CD4+ T cells are vulnerable to apoptosis by FasL from epidermal keratinocytes. Langerhans cell is decreased in numbers in the epidermis and its trafficking to lymph nodes is enhanced

that enhanced LC migration to draining lymph nodes, and that the chronic nature of As-BD might result from enhanced Th1 responses with dysregulated LC trafficking (manuscript in preparation).

6. Conclusions

Bowen's disease is the most common form of arsenic-induced skin cancers. As-BD is characterized by its chronicity, multiplicity and predisposition in sun-spare skin. Patients with As-BD are often defected in their cellular immunity (**Figure 1**). CD4+ T cells is quantitatively reduced in people with As-BD. CD4+ T cells from arsenic-exposed individuals are less susceptible to apoptosis due to an impaired TNF-alpha-TNFR loop. However, once CD4+ T cells gain access the As-BD lesions, FasL from epidermal keratinocytes in As-BD induces selective CD4+ cell apoptosis. Notably, there is also a decrease in numbers and aberrant trafficking of Langerhans cells in As-BD. Thus, arsenic differentially affects systemic and localized immunity in arsenic skin carcinogenesis. In chemical carcinogenesis, the attribute of chemical immunology should be considered.

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Antioxidant Potential Some Medicinal Plants of Central India

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ABSTRACT

Cellular damage or oxidative injury arising from free radicals or reactive oxygen species (*ROS*) *now appears the fundamental mechanism underlying a number of human neurodegenerative disorder, diabetes, inflammation, viral infections, autoimmune pathologies and digestive system disorders. Free radicals are generated through normal metabolism of drugs, environmental chemicals and other xenobiotics as well as endogenous chemicals, especially stress hormones* (*adrenalin and noradrenalin*). *Accumulated evidence suggests that ROS can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants. In this review, research on the antioxidant potential of some medicinal plants of origin of Central India is considered.*

Keywords*: *Medicinal Plants, Antioxidant Activity, Chemoprevention, Neurodegenerative Diseases, Central India.

1. Introduction

Considerable evidence have accumulated to implicate cellular damage arising from reactive oxygen species (ROS), at least in part, in the etiology and pathophysiology of human diseases such as neurodegenerative disorders such as neurodegenerative disorders (e.g. Alzeimer disease, Parkinson disease, multiple schlerosis, Down's syndrome), inflammation, viral infections, autoimmune pathologies, and digestive system disorders such as gastrointestinal inflammation, viral infections, autoimmune pathologies, and digestive system disorders such as gastrointestinal inflammation and ulcer [1-3]. In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, by bacterial leucocytes, through xanthine oxidase activity, atmospheric pollutants, and from transitional metal catalyst, drugs and xenobiotics. In addition, chemical mobilization of fat stores under various condition such as lactation, exercise, fever, infection and even fasting, can result in increased radical activity and damage, in particular, to the immune and nervous systems, while the stress hormones (adrenalin and noradrenalin) secreted by the adrenal glands under conditions of continuation and excessive emotional stress, are metabolized in to simpler, albeit, free radical molecules.

Free radical or oxidative injury now appears the fundamental mechanism underlying a number of human neurologic and other disorders [4]. For instance in diabetes, increased oxidative stress which co-exist with reduction in the antioxidant status has been postulated: Oxygen free-radical can initiate per oxidation of lipids, which in turn stimulates glycation of protein, inactivation of enzymes and alteration in the structure and function of collagen basement and other membranes, and play a role in the long term complication of diabetes [5-7]. Similarly, in carcinogenesis, reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma [8]. Hence, therapy using free-radical scavengers (antioxidants) has potential to prevent, delay or ameliorate many of these disorders [9]. Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis [3,8,10-15]. Similarly evidence also exit to demonstrate the chemo preventive capacities of ethno botanicals and components of vegetable diets with free-radical scavenging potential on ulcers [16], diabetes [5], memory and cognitive function [17], Alzheimer's disease [17,18], age-related neurological dysfunction [19], cardiovascular

and renal disorders [20].

In the modern word it has been realized the herbal drugs strengthens the body system specifically and selectively without side effects. The importance of traditional herbal medicinal system has now gained vital importance in developed countries has been briefly described. The herbal medicinal practices adopted by traditional medicine-man in Central India for cure of various diseases occurring in tribal pockets. India is blessed with rich and diverse heritage of cultural traditions. These traditions are associated with use of wild plants as medicinal herbs. The use of medicinal herbs is still a tradition adopted by ethnic communities who are living in undulating plains and at foothills of dense forest. The Central India comprises states like Madhya Pradesh, Chhatisgarh, Maharastra, Orissa and Jharkhand. The ethnic people of thisregion are Baiga, Bhariya, Bhil, Gond, Hill korwa, Birhor, Khairwar, Rawat and Sahariyas. They use wide range of wild plants for their health care [21].

The traditional herbal healer therapies contain many medicines for one ailment. Out of the various medicines, one is selected by the herbal healer against a particular disease according to the symptoms and secondary effects. Several plants are identified and used against one disease and are used according to their availability in the region. Some of the plants commonly used by tribal in Central India for prominent diseases are recorded during the various surveys.

2. Central India Plants

In spite of tremendous advances in modern medicine no effective drugs are available, which stimulate liver functions and offer protection to the liver from damage of help to regenerate hepatic cells 1) In absence of reliable liver-protective drugs in modern medicine, a large number of medicinal preparations are recommended for

Table 1. Some medicinal plants of central India having good antioxidant potential

Name of plant	Part Studied	Type of Assay	Active component(s)
Acorus calamus	Rhizomes	In vitro	α-asarone, Alkaloids [25]
Aegle marmelos	Leaves	In vitro	Alkaloids, Terpenoids, Saponins [26]
Aloe vera	Leaf	In vitro	Vitamin A,C,E, Carotenoids [27]
Andrographis paniculata	Whole plant	In vitro/ in vivo	Diterpenes, Lactones, [24]
Carica papaya	Leaves	In vitro/ in vivo	Terpenoids. Saponins, Tannins [28]
Cassia fistula	Bark	In vitro	Flavonoids [29]
Curculigo orchioides	Rhizomes	In vivo	Alkaloids, Flavonoids [30]
Cyperus rotundus	Rhizomes	In vitro	Saponin, Sesquiterpenoids, [31]
Dalbergia sisoo	Leaves and flower	In vitro	Tannins [32]
Emblica officinalis	Seeds	In vitro	Vitamin C, Tannins [33]
Ficus bengalensis	Aerial root	In vitro	Flavonoids, Tannins [34]
Hemidesmus indicus	Stem	In vitro	Alkaloids, Glycosides [25]
Magnifera indica	Stem bark	In vitro	Reducing sugar, Flavonoids [28]
Momordica charantia	Fruit	In vitro	Alkaloids, Saponin [35]
Moringa olifera	seeds	In vitro/ in vivo	Glycosides [34]
Ocimum sanctum	Leaf	In vitro/ in vivo	Carotenoids, Ascorbic acid [33]
Plumbago zeylanica	Root	In vitro	Alkaloids, Glycosides [25]
Psidium guajava	Leaves	In vitro	Flavonoids, Limonoids [28]
Solanum nigrum	Fruit	In vitro	Carotenoids, Ascorbic acid [33]
Syzygium cumini	Leaf	In vitro	Triterpenoids, Ellagic acid [36]

the treatment of liver disorders 2) and quite often claimed to offer significant relief. Attempts are being made globally to get scientific evidences for these traditionally reported herbal drugs. *Amorphophallus campanulatus* Roxb. (ACR) (family: Araceae), is aperennial herb with rounded tuberous root stock (corn). The plant is widely distributed in Central India. The tuberous roots of the plant have been used traditionally for the treatment of piles, abdominal pain, tumors, enlargement of spleen, asthma and rheumatism 3) The tuberous roots of the plant also been possess tonic, stomachic and appetizer properties [22].

In 2002 at the international Aloe Science Council (IASC) Annual Conference, Vinson Joe presented evidence from a human chemical study, that the bioavailability of antioxidant supplement vitamins C and E was increased by over 200 percent when taken Aloe Vera gel.

The decreased activity of antioxidant molecular along with elevated lipid peroxide levels in diabetic rat could probably be associated with oxidative stress and/or decreased antioxidant defense potential. The reversal in their content following treatment may be due to decreased oxidative load. The *Aloe barbadensis* leaf extracts may also act by either directly scavenging the reactive oxygen metabolites, due to the presence of various antioxidant compounds or by increasing the synthesis of antioxidant molecules [23].

Andrograph paniculata is a perennial herb widely cultivated in Central India and traditionally used as febrifuge, tonic, stomachic and anthelmintic. Andrographis forms the principal ingredient of several pharmaceutical preparations and household medicines too. Modern pharmacological studies have demonstrated its hepatoprotective, antithrombotic, anti-inflammatory, immunostimulant, antimalarial, antihyperglycemic and cardioprotective properties.

The role of free radicals generated oxidative stress in isoproterenol-induced myocardial ischemic injury is well established. Several herbal drugs possessing antioxidant activity have been demonstrated protective in the isoproterenol-induced ischemic injury of the myocardium. Andrographis is one of the plants used as antioxidant and acclaimed to provide benefie in cardiovascular diseases in traditional literature [24].

3. Conclusions

Considering the enormous biodiversity resources of the Central India, and the high incidence of diseases with oxidative damage as their etiological factor in this area, a total 20 medicinal plants investigated from Central India. Active principle responsible for the antioxidant properties were also identified. (**Table 1**).

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Feasibility of Performing Sentinel Lymph Node Biopsy (SLNB) after Mastectomy: A Case Report

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ABSTRACT

***Introduction*:** *Previous mastectomy remains a contraindication to SLNB as normal drainage patterns of the breast can be disturbed. Patients diagnosed with DCIS on core biopsy and later found to have microinvasive or invasive carcinoma at the time of mastectomy are deprived of the opportunity for SLNB and need to undergo axillary dissection. We explored the option and feasibility of performing SLNB in a 39-year-old female who underwent a simple mastectomy without axillary sampling for extensive DCIS and later found to have microinvasive ductal carcinoma on permanent pathology.* ***Results*:** *Lymphatic mapping using subdermal injection of 99mTc-labeled sulfur colloid and blue dye led to the identification of five SLNs. Histopathologic examination showed no metastasis.* ***Conclusion*:** *SNLB is feasible in this setting. However, before its use is routinely adopted, its feasibility and accuracy has to be demonstrated in larger numbers of patients in whom a negative SLNB is followed by a completion axillary dissection.*

***Keywords*:** *Sentinel Lymph Node Biopsy, Lymphatic Mapping, Mastectomy, Lymphoscintigraphy, Axillary Lymph Node Dissection*

1. Introduction

Axillary lymph node status remains the single most important prognostic factor for invasive, early-stage breast cancer. Currently, SLNB is routinely performed in clinical practice but the indications for SLNB continue to evolve. In the 1990s, SLNB was strictly limited to patients with small invasive tumors and clinically negative axillae. With increased experience and the widespread use of the technique, indications for SLNB have since expanded to most patients with clinically node-negative, operable breast cancer. More recently, the suitability of SLNB has also been considered in the setting of intraductal carcinoma (DCIS) and DCIS with microinvasion. However, previous mastectomy remains a contraindication to SLNB as it has been suggested that the normal drainage patterns of the breast can be disturbed shortly after surgery or radiotherapy and that prior excisional biopsy can alter lymphatic drainage in up to 70% of the patients. Thus, patients who are diagnosed with DCIS on core needle biopsy and are later found to have microinvasive or invasive carcinoma after mastectomy are deprived of the opportunity for SLNB and need to undergo an axillary dissection. We evaluated the feasibility of SLNB in a patient who was referred to our center following simple mastectomy for a large area of DCIS (based on core biopsy) and who was later found to have microinvasive breast cancer on permanent pathology.

2. Case Report

A 39-year-old Caucasian female underwent a simple mastectomy for extensive DCIS, one month prior to presentation to our center. No histologic examination of the axilla was performed. She was found to have microinvasive ductal carcinoma on permanent pathology and was recommended to undergo axillary dissection but wanted to explore alternatives, including SLNB. We proceeded with lymphatic mapping and SLNB. Lymphatic mapping was performed using subdermal injections of 99 m Tc-labeled sulfur colloid in the skin flaps both above and below the previous mastectomy scar. Subdermal injections of blue dye took place intraoperatively in the same fashion and the area was massaged for five minutes. Axillary lymph nodes were designated as sentinel nodes (SLN) and biopsied if they were clinically palpable in surgery, radioactive, blue or any combination of the above.

Five SLNs were identified. One was hot (10-second count of 169), one was blue but not hot and three were

only clinically palpable but not clinically suspicious. Histopathologic examination showed no metastasis. Given the low-risk for axillary metastasis, no completion axillary dissection was performed.

3. Discussion

Lymphatic mapping and SLNB are well-established tools for staging primary breast carcinoma and minimizing morbidity associated with level I and II axillary node dissection (ALND). Axillary SLNB is currently considered the gold standard for staging the axilla in clinically node-negative women [1]. Several investigators have confirmed the hypothesis that lymphatic drainage of a breast cancer can be identified and traced to the sentinel node during surgery, and that the histologic status of the sentinel node accurately predicts the pathologic status of the entire axilla [2,3-9]. The sentinel lymph node concept for early breast cancer has been well validated in many studies. [4,5,7-9,10-12] Although initially lymphatic mapping was performed by peritumoral injections of radioisotope or blue dye, more recently, the intradermal injection route has been shown to result in a significantly greater frequency of localization, decreased time to first localization on preoperative lymphoscintigraphy, and decreased time to harvest the first SLN [13].

To be anatomically and oncologically effective and to correctly predict the histological status of the axilla, SLNB requires the presence of an intact lymphatic flow from the site of the primary tumor (or recurrence). Previous mastectomy partially and/or temporarily interrupting and modifying the lymphatic flow renders it theoretically more difficult to correctly identify the SLN. In such a situation the concept of SLN itself could be questioned and ALND is always considered mandatory, even in cases of small limited recurrences with low risk of axillary involvement. Thus, following mastectomy, several questions need to be addressed relative to the potential value of pursuing a SLNB. Where is the chest wall lymphatic drainage anatomically directed? And if a new SLN exists, is its predictive value biologically comparable to that of SLNs from an intact breast? [14]

The breast drains through common afferent lymphatic channels to common axillary nodes [15]. This lymphatic pathway supports the hypothesis that the breast drains as a single unit, and therefore the location of the primary tumor within the breast does not affect the location and number of the sentinel nodes with the exception of tumors located deep in the breast, which can drain either to the axilla or to the internal mammary chain via the deep retromammary lymphatic pathway.[16] Thus, the variable which determines the number and location of SLNs in the axilla is the number of afferent lymphatic pathways to the axilla rather than the number or site of injections. This evolution in the SLN concept has also been considered the rational basis on which SLNB may be applied to patients with a multicentric disease [17]. A mastectomy can interrupt and strongly modify the lymphatic flow of the thoracic wall, but the physiological restoring of the anatomy of the lymphatic drainage renders the obstacle only temporary. In fact, when an adequate period of time elapses between the mastectomy and the local recurrence, the lymphatic network has time to be physiologically re-built and a new lymphatic "bridge" can connect the thoracic wall to the afferent loco- regional lymph nodes. Such a postoperative "collateralization" of lymphatics occurs as a compensatory mechanism. Presumably, the once predictable nodal drainage architecture becomes distorted, but this need not necessarily preclude the presence of a loco-regional SLN in such cases. In fact, a novel SLN has been documented in a case of small breast recurrence after breast conserving surgery (BCS) and complete axillary dissection (CAD) (the new SLN was in the contralateral axilla) [18], after partial axillary dissection (PAD) (the new SLN was in the ipsilateral axilla) [19], and after previous SLNB (again, the new SLN was in the ipsilateral axilla) [20]. These considerations, introducing a new dynamic concept of SLN (not "one SLN for ever" but "always a new SLN"), have convinced authors to propose and successfully perform lymphoscintigraphy and SLNB after BCS and previous CAD [18], PAD [19] and SLNB [20].

In women with a recurrent or second ipsilateral breast carcinoma and history of previous breast and axillary surgery lymphoscintigraphy is successful in identifying a SLN in 69% of patients. Lymphatic drainage patterns —including drainage across the midline of the thorax in 25% of patients—vary widely. Lymphoscintigraphy in these patients is especially useful for preoperative planning to ensure inclusion of potential sentinel nodes in the contralateral axilla or supraclavicular and infraclavicular regions within the operative field. Although internal mammary nodes are not routinely biopsied, identification of internal mammary nodes may be useful for prognostication and radiation treatment planning1. Using dual tracers consisting of blue dye and lymphoscintigraphy with technetium radiocolloid affords surgeons an opportunity to observe actual native and collateralized lymph node drainage architecture in real-time. Subsequent identification of nodal uptake in any nodal region for patients with primary or recurrent breast malignancies may provide pertinent clinical data relevant to optimal staging and treatment [18]. The same theory of lymphatic dynamics following BCS for patients with primary or recurrent breast malignancies could be applied for patients following mastectomy. While there is paucity in the literature of previous experiences with SLNB following mastectomy, the same concept of lymphatic distortion and, hence, the same potential challenges with SLNB apply.

4. Conclusions

Subdermal injection of both radioisotope and blue dye led to the identification of axillary SLNs in a patient who

had recently undergone a simple mastectomy. This case demonstrates the potential feasibility of the procedure in this setting. However, before its use is routinely adopted, its feasibility and accuracy has to be demonstrated in larger numbers of patients in whom a negative SLNB is followed by a completion axillary dissection.

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Microangiopathic Hemolytic Anemia and Diffuse Bone Metastasis by Signet Ring Cell Adenocarcinoma

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ABSTRACT

Microangiopathic hemolytic anemia (*MAHA*) *is a rare paraneoplastic syndrome and is typically associated with gastric adenocarcinoma. We report a* 47*-year-old woman who presented with asthenia, lower back pain and bleeding. Twelve years ago the patient underwent total gastrectomy due to gastric adenocarcinoma and achieved complete remission. The patient was diagnosed with MAHA and diffuses bone metastasis of signet ring cell adenocarcinoma of unknown origin and was treated successfully with polichemotherapy based on cisplatin and* 5*-fluorouracil.*

Keywords: *Microangiopathic Haemolytic Anemia, Gastric Cancer, Paraneoplastic Syndrome, Chemotherapy, Signet Ring Cell Adenocarcinoma*

1. Introduction

MAHA was first described in 1962 by Brain *et al.* [1] and is an uncommon haematological disorder which can appear in different diseases (thrombotic thrombocytopenic purpura, haemolytic ureamic syndrome, vasculitis and paraneoplastic syndrome in cancer) [2]. It is defined as a severe haemolytic anemia with negative Coombs test and fragmentation of red cells in the peripheral blood smear. MAHA occurs in patients with metastasized signet cell carcinoma of stomach but it's been described in breast and lung carcinoma [3]. The pathogenesis of cancer-related MAHA is not well understood. Typically, it's associated to diffuse bone metastasis by signet ring cell adenocarcinoma. MAHA can be the first manifestation of metastatic carcinoma and is associated to extremely poor prognosis [4,5]. Sometimes is the first sign of recurrence after a curative surgery even many years later [6,7].

In this report we describe the case of a 47-year-old woman with a medical history of gastric cancer twelve years ago who was diagnosed with MAHA and massive infiltration of bone marrow by signet cell carcinoma. After careful workup the primary site could not be identified. The patient responded dramatically to polichemotherapy and long-term partial response was achieved.

2. Case Report

A 47- year-old woman was referred to our service with a medical history of 8 months of lower back pain. During the last week she referred asthenia, vaginal bleeding, epistaxis and bulky bruises widespread. Twelve years ago the patient was diagnosed with gastric adenocarcinoma. She underwent total gastrectomy and achieved a R0 resection (pathological stage pT2pN0M0). No adjuvant treatment was delivered.

At the emergency room the patient showed pale skin, tachycardia at 110 bpm. No other relevant signs were found at physical examination, ECOG=3. Laboratory work-up yielded the following results: haemoglobin 4,9 g/dL, white blood cells (WBC) 8,400/μL with 39,6% neutrophils and 36,2% lymphocytes, platelets 9.000/μL, total bilirubin 2.1 mg/dL, GOT 69 U/L, GPT 57 U/L, alkaline phosphatase 3.494 U/L, LDH 3.863 U/L, renal function, electrolytes and the rest of the values were normal. Peripheral blood smears showed a leukoerythroblastic reaction with 33% eritroblasts and many schistocytes. Direct and indirect Coombs test: negative. Bone scan showed multiple bone metastases in the whole spine, pelvis, ribs and calvaria. CT body scans showed density abnormality of the perianastomotic tissue suggestive of local tumor recurrence and diffuse bone metastasis. Up-

per GI endoscopy, colonoscopy, mammography and abdominal ultrasound were normal. Bone marrow biopsy disclosed metastasis of a signet ring cell adenocarcinoma. Inmunohistochemical staining was positive for CA19.9, CEA, cytokeratins (AE1, pancK, 8,18,19) and negative for citokeratins AE3, CD45, CD20, CD30, ALK1, CD68, CA125, c-erb-2, estrogen receptor, progesterone receptor, bcl-2, p53. The Ki67 rate proliferation was low. PET scan was not available.

Therefore a diagnostic of metastatic signet cell carcinoma of probably gastric origin with paraneoplastic MAHA was established and the patient was treated with multiagent chemotherapy cisplatin 80 mg/m^2 intravenous (iv) on day 1 and 5-FU 1.000 mg/(m^2·day) continuous infusion on days 1-5, zoledronic acid and intensive blood and platelet transfusions. After the first cycle of chemotherapy, the vaginal bleeding and epistaxis stopped and the hemoglobin level stabilized (> 8 g/dL) and platelet increased over 30.000/μL. Six courses of chemotherapy were delivered and clinical and laboratory response was evident (haemoglobin 11 g/dL, WBC 3.900 with 54% neutrophils, normal platelets, alkaline phosphatase 340 U/L,LDH 522 U/L). A bone scan and CT scan showed a partial response in the number and intensity of bone lesions. Chemotherapy was then stopped and the patient started a follow-up program in our service. Three months later, the bone metastases progressed and the patient was treated with antialgic radiotherapy over L4-L5 (total dose 20 Gy). She received second line treatment with FOLFIRI, four cycles were delivered with progression of disease. Third line multiagent chemotherapy DCF (docetaxel 75 mg/m^2 iv day 1, cisplatin 75 mg/m^2 iv day 1, 5-FU 750 mg/m^2/day continuous infusion on days 1-5) with a 20 % dose reduction was started. She received 4 cycles with excellent clinical, biochemical and radiological response. After the fifth cycle, the patient came to the emergency room with an evident progression of the disease with severe pain, grade IV anemia, leucopenia and thrombopenia and radiological progression of the disease. The patient finally died due to bleeding and hypovolemic shock nineteen months after diagnosis. Unfortunately autopsy was not performed.

3. Discussion

Chemotherapy is the only treatment that can control the cancer and the paraneoplastic syndrome in this clinical setting and should be started as soon as possible. Due to the clinical findings and the history of gastric cancer, we decided to treat the patient as relapse of gastric cancer. Several case reports have shown good responses to chemotherapy, although there is no evident standard of care first-line chemotherapy [2]. We decided to use a combination regimen of two drugs instead of a triplet due to the poor performance status of the patient and the better toxic profile of the doublet [8]. We avoided regimens with epirubicin and docetaxel because of the myelotoxicity of these drugs. An alternative approach is the combination of oxaliplatin and fluoropyrimidines that have shown in phase III clinical trials to be less toxic and at least as effective as the combinations with cisplatin [9,10]. Despite the extremely severe thrombocytopenia, this should not be considered a contraindication of chemotherapy. The good partial response to chemotherapy and the long term survival achieved with chemotherapy show chemotherapy should be considered in all patients in this setting despite a poor performance status or severe cytopenias.

Positron emission tomography (PET) scan is a useful diagnostic test in metastasis of unknown origin. PET is a useful procedure both to detect the primary tumor and to identify additional sites of metastatic disease. PET detects more sites of metastasis than other modalities, and in 20% to 40% of cases it discloses the site of the primary tumor [11]. Its exact role is yet to be defined because of a lack of prospective clinical trials comparing the performance of PET with conventional diagnostic test. Unfortunately, in this clinical case PET scan was not performed due to we lacked this technique at the time of the patient was diagnosed.

Gastric cancer usually relapses in the first three years after surgery [12], however similar clinical cases have been reported with long periods of time between the primary tumor and the recurrence [5,6]. Some authors have suggested the origin of the metastasis in dormant tumor cells in the bone marrow and lymph ducts of the gastric wall in spite of normal gastric mucosa [6]. Our clinical case may support this hypothesis, however it is unclear why tumor cells remain dormant for as long as 12 years and which mechanism let them grow.

4. Conflict of Interest

The authors declare that they have no conflict of interest relating to the publication of this manuscript.

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Proteomic Profile Modification of Anaplastic Medulloblastoma after *in*-Vivo Radiotherapy: A Case Study

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ABSTRACT

Medulloblastoma (*MDB*) *is an aggressive tumor of Central Nervous System* (*CNS*). *Radiotherapy after radical surgery has an important role in treatment of standard and high risk patients and is followed by intensive chemotherapy. To explore modifications of protein expression induced by in vivo radiotherapy proteomic analysis was performed on a case of Anaplastic MDB.* 2*D-gel electrophoresis and MALDI-TOF mass spectrometry detected qualitative differences of protein expression in Anaplastic MDB at diagnosis and in relapse after radiotherapy. Relevant proteomic data were confirmed by western blot and Real-Time PCR analysis, validating the presence of Sthatmin* 1 (*STMN*1), *Heat shock protein* 60 (*HSP*60), *HSP*27 *and Disulfide Isomerase* (*ER*60) *among the six proteins present in both samples. The most relevant modification induced by radiotherapy was a drastic reduction of the total number of proteins* (60.6%) *and the appearance of few new proteins. The modifications and the striking simplification of proteins expressed by the tumor after radiotherapy may allow to tailor subsequent chemotherapy on a rational basis. A proteomic guided chemotherapy may be of great benefit to patients.*

Keywords*: *Proteomics, Anaplastic Medulloblastoma, Radiotherapy

1. Introduction

Medulloblastoma (MDB) is an aggressive tumor of central nervous system (CNS) located in the cerebellum and represents the more frequent and studied malignant tumor of CNS of paediatric age [1]. MDB less frequently affects adult patients [2,3]. With modern protocols survival is high (70-80%) but neuropsychological damages on developing brain may be severe. Radiotherapy is one of the relevant causes of therapy related brain damage. The aim of modern protocols is to grade tumor aggressiveness relying on solid prognostic factors. The intensity of treatment may be modulated by risk group (low-standard-high). Such an approach may limit the use of radiotherapy to less favorable groups of patients and protect infants and young children from radiotherapic damages [4].

Proteomic studies on MDB were mainly limited to cell lines DAOY and D283 which shared Ded protein, an antiapoptotic principle [5]. Modifications induced in vivo by radiotherapy have not been investigated with a proteomic approach. The presented case offered the unique opportunity to investigate an Anaplastic MDB at diagnosis and at relapse after sole radiotherapy.

2. Materials and Methods

2.1 Patient

The patient, a 26 year old white male, presented with a large tumor of the cerebellar vermis (**Figure 1(a)**) which was totally resected (**Figure 1(b)**). Histological diagnosis was Anaplastic MDB, according to WHO 2007 classification [1]. The patient underwent radiotherapy, which delivered 54 Gray on the whole neuraxis with regression of clinical symptoms. Eight months later a local relapse developed (**Figure 1(c)**) and a second neurosurgery was performed.

2.2 Proteomics

2D-electrophoresis using pH range of 3-10 (Bio-Rad) was performed as previously described [6] on snap frozen tissue of the primary tumor to detect a complete pro-

teomic profile of the sample. Since the majority of proteins were included in the 4-7 pH range, to analyze the proteins involved in radiotherapy response, further analysis of primary tumor and its relapse was limited to such range. More than 450 detectable protein spot were found on primary tumor and 270 in the relapse gel with the aid of image analysis software PDQuest version 7.2 (Bio-Rad). As previously described [7], the spots excised from 2D-electrophoresis Colloidal Comassie stained gels were destained (in 50% ACN and 5 mM NH_4CO_3), dehydrated in 100% ACN and digested overnight with 5 µl trypsin solution (0,1 mg/ml trypsin and 5 mM NH_4CO_3). The mass spectrometer used in this work was a MALDI-TOF (MALDI micro MX, Waters, MA, USA) with a delayed extraction unit. Peptide spectra were obtained in reflection mode in the range 800-3000 Da. Database searching was performed using the 25 most intense measured peptide masses against the Swiss-Prot database (one missed cleavage with the trypsin enzyme selected, oxidation of methionine as potential variable modification, peptide tolerance of 100 ppm, taxa human) using the free search program MASCOT (http://www.matrixscience.com).

Only protein identifications with significant Mascot score ($p < 0.05$) were taken into consideration.

2.3 Western Blotting

The presences of Stathmin 1 (STMN1) was investigated by western blot. Protein samples (20 µg) were separated in a 10% polyacrylamide gel and transferred to nitrocellulose membrane. STM1 rabbit polyclonal antibody (Cell Signaling) was diluited using manufacturer's instruction and incubated overnight at 4˚C. After incubation with the appropriate secondary antibody, immunoreactive bands were detected with an enhanced chemiluminescence (EuroClone). The membranes were after stripped and reprobed for β-Tubulin (rabbit monoclonal, 9F3; Cell Signaling, 1:1000 diluition) as a protein loading control.

2.4 Real Time PCR

The presence of Heat Shock Proteins 27 (HSP27), HSP60, ER60 in both tumor samples was investigated by Real-Time PCR analysis in order to validate proteomic findings. Ribosomal protein (S14) was used as reference gene to normalize cDNA across samples. Primers were designed using Primer 3 with the Beacon Designer Software (PREMIER Biosoft International) for: 1) HSP27 forward GCTGACGGTCAAGACCAAGG-3' and reverse 5'-ATGTAGCCATGCTCGTCCTG-3'; 2) HSP60 forward 5'-GCTGAAGATGTTGATGGAGA-3' and reverse 5'-TTGTCACCAAACCCTGGAGC-3'; 3) ER60 forward 5'-TTCCTGTTGCTATCAGAACTG3' and reverse 5'-ACCTCTCCAGAGCCTTCCCA-3'. Real-time-PCR was performed with the ABI PRISM 7900 Ht Applied BioSystem instrument.

3. Results and Discussion

The present case offered the unique opportunity of study the original tumor (**Figure 1(a)**) that underwent radical resection (**1(b)**) and relapse (**1(c)**) after radiotherapy. MRI control scan, two weeks after second surgery, detected dorso-lumbar leptomeningeal seeding. Chemotherapy was started with Cisplatinum and Vincristin. Despite chemotherapy the patient died few weeks later.

To investigated radiotherapy response proteomic analysis of primary tumor and relapse was performed. **Figures 1 (d)** and **(e)** demonstrated that these two tissue samples (Anaplastic Medulloblastoma at diagnosis and at relapse) presented remarkable different proteomic patterns, with striking difference in the distribution and number of proteins or spot intensity. From gel comparison sixty-one proteins (**Tables 1-3**) appeared differentially expressed between primary tumor and its relapse after radiotherapy. Among the proteins identified, 37 (60.6%) proteins were only present in the original tumor and lost after radiotherapy (**Table 1**), and 18 (29.5%) were only found in the relapse tumor after radiotherapy (**Table 2**); 6 (9.8%) proteins were actually present in both samples (**Table 3**). Several protein biologically related to MDB growth and aggressiveness were present only in the primary tumor: these included MEIS1, which has DNA-binding property and a role in embryogenesis and in CNS development [8], Annexin VIII involved in regulation of process of apoptosis, some Heat Shock Proteins (HSP70 protein 5 and 8) and several enzymes or enzyme inhibitors (Peroxiredoxin-2, Alpha-1-antiproteinase, alfaenolase, Cyclin dependent kinase 9, GTPase activating protein, etc). An impressive modification of the protein repertoire was induced by radiotherapy. Only six proteins were expressed in both tumor samples (**Table 3**). From these STM1, HSP60, HSP27 and ER60 were further investigated. The presence in both samples of STMN1 was confirmed by western blot (**Figure 2(a)**), while that of HSP27, HSP60 and Disulfide Isomerase by Real-Time PCR. **Figure 2(b)** illustrated the ratio between messenger RNA levels in Anaplastic MDB and levels in MDB after radiotherapy. In agreement to proteomic data, HSP27, HSP60 and ER60 proteins were expressed in both tumor with the similar levels. These conserved proteins, which possibly represented an essential protein asset linking the original tumour to the post radiotherapy relapse, were considered of biological relevance. STM1, a cytosolic phosphoprotein involved in cell proliferation and mobility, has been reported as a marker of aggressiveness in MDB [9]. HSPs originally described as chaperonines also play a role in tumor development and progression in several tumors [10] with possible modulated influence depending on histotype. ER60 showed a role in protein folding in Endoplasmic Reticulum and, with its precursor ER57, interacts with important signalling targets and III

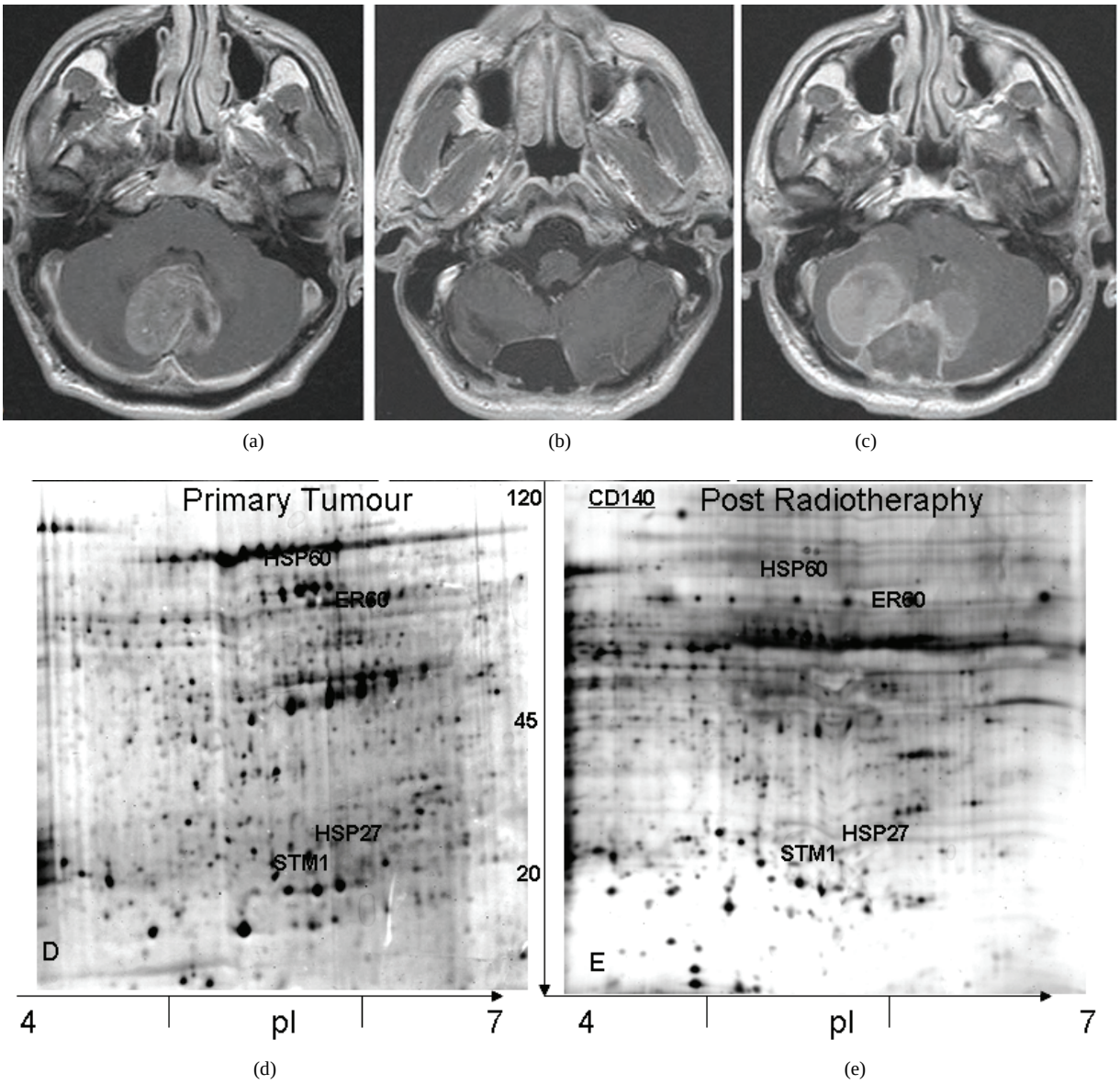

Figure 1. NMR images of the patient. (a) At diagnosis T1- weighted NMR demonstrating a large vermian mass; (b) After radical surgery no residual tumor is detectable; (c) At relapse tumor infiltrates cerebellar hemispheres. Comassie blue stained 2D-electrophoresis patterns of Anaplastic MDB. 2D-electrophoresis was performed on immobilized pH 4-7 strips, followed by SDS-PAGE on 10%, 7cmx8cm gels. (d) 2D-electrophoresis pattern of primary tumor shows a large number of proteins. (e) At relapse tumor 2Delectrophoresis demonstrates an impressive reduction of the protein profile. Proteins validate by other techniques are marked by name. Total spots excised from D and E are reported in details in Table 1

beta Tubulin [11].

Radiotherapy has a key role in the treatment of MDB in children as well as in adult patients [12,13] and almost all protocols for high risk patients include radiotherapy treatment as a second step, after radical surgery, followed by chemotherapy. Tumoral cell death induced by radiotherapy is obtained by DNA damages but relevant differences in radiosensitivity were observed in tumors of different histotype and the factors conferring resistance to radiotherapy are not completely understood [14]. MDB is a radiotherapy sensitive neoplasia but mechanism that modulate cell resistance in vivo are not well known. A proteomic approach has been utilized on epithelial cancers to correlate protein expression to response to radiotherapy [15,16]. These studies on colonic or breast cancer did not analyze protein expression modifications on RT treated tumors in vivo. Recently, utilizing a murine model of MDB and MDB cell lines, tumor cell modifications after the exposure to a course of total body radiation were investigated [17]. This study re-

Table 1. Proteins identified by MALDI-TOF only on the sample of the primary tumor

Entry	Description	MW	pI	Score	Peptides	*C %
P38646	75 kDa glucose regulated protein GRP 75	73634	5,7795	5	9	54
P11021	Heat shock 70 kDa protein 5	72288	4,8748	10	34	51
Q9P157	Serum albumin	71317	5,92	12	24	102
P11142	Heat shock cognate 71 kDa protein	71082	5,37	10	23	88
P11142	Heat shock 70 kDa protein 8	70854	5,1998	12	15	54
P10809	60 kDa heat shock protein, mitochondrial	61187	5,7	6	18	59
P17987	protein 1 subunit alpha	60305	5,7127	12	4	56
P13645	Keratin, type I cytoskeletal 10	59020	5,13	9	20	80
P05187	phosphatase placental type precursor	57917	5,8326	7	4	67
P08670	Vimentin	53619	4,8629	10	14	62
P61980	nuclear ribonucleoprotein K	50944	5,2203	7	16	57
Q71U36	Tubulin alpha-1A chain	50788	4,94	6	24	59
Q13885	Tubulin beta-2A chain	50274	4,78	7	22	70
Q6GMP2	Alpha-enolase	47481	7,01	14	35	123
Q86U18	Alpha-1-antitrypsin	46878	5,37	16	43	187
P01009	Alpha 1 antiproteinase	46707	5,2405	5	11	51
P07339	Cathepsin D	45037	6,1	8	27	80
O00470	protein Meis1	42988	5,8502	7	4	41
P50750	Cyclin dependent kinase 9	42750	8,9866	5	5	65
P50219	protein HB9	40726	7,4603	6	4	61
Q9UNZ2	cofactor p47	40548	4,8067	9	14	52
P62140	Serine/threonine-protein phosphatase PP1-beta subunit	37961	5,84	4	14	47
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	37464	8,97	7	24	73
P13928	A8 Annexin VIII	36855	5,436	7	5	45
P04406	Glyceraldehyde-3-phosphate dehydrogenase	36201	8,57	10	44	65
Q15181	Inorganic pyrophosphatase	33095	5,54	7	34	99
O94760	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	31444	5,53	10	40	116
P60174	Triosephosphate isomerase	26938	6,45	13	67	198
P09936	Ubiquitin carboxyl-terminal hydrolase isozyme L1	25151	5,33	10	51	142
P43487	GTPase activating protein	23295	5,0228	7	10	44
Q9UC36	Heat shock protein beta-1	22826	5,98	6	32	82
P32119	Peroxiredoxin-2	22049	5,66	6	35	94
P30086	Phosphatidylethanolamine-binding protein 1	21158	7,01	10	55	154
P23528	Cofilin-1	18719	8,22	6	42	73
P15531	Tumor metastatic process associated protein	17137	5,7662	7	11	64
Q549N7	Hemoglobin subunit beta	16102	6,75	9	63	158
P00441	Superoxide dismutase Cu Zn	15925	5,6655	8	5	44

*C: coverage

Table 2. Proteins identified by MALDI-TOF only on the post-radiotherapy sample

Entry	Description	MW	pI	Score	Peptides	*C %
P16234	CD140a antigen	122591	4,8768	8	12	55
P22681	ubiquitin protein ligase CBL	99583	6,0773	5	4	47
Q86VS8	homolog 3 hHK3	83074	4,9314	6	5	55
P52888	oligopeptidase Endopeptidase 24 15 MP78	78789	5,6409	8	6	43
Q96BP3	isomerase domain and WD repeat containing protein 1	73527	6,7214	7	6	41
P16519	convertase 2 precursor NEC 2	70520	6,0289	7	5	62
P35398	receptor ROR alpha	62995	5,9366	7	5	76
O43278	Hepatocyte growth factor activator inhibitor type 1	58360	5,8478	5	4	70
Q5VTY9	Melanoma antigen recognized by T cells 2	57274	6,9218	6	5	61
P04217	precursor Alpha 1 B glycoprotein	54238	5,4948	8	9	57
Q92733	protein PRCC Pap.renal.cell.carc.Trans. Ass.gene protein	52385	4,8268	8	4	40
Q9UC06	finger protein 70	50769	8,2009	7	6	42
P31943	nuclear ribonucleoprotein H hnRNP H	49198	5,8513	9	21	50
Q5JT82	factor 17 Zinc finger protein 393	42550	6,2813	5	5	68
Q12829	protein Rab 40B	30936	9,936	5	6	79
O95865	dimethylaminohydrolase 2	29625	5,5895	8	9	68
Q04760	Aldoketomutase	20706	5,0781	5	7	61
P09382	Putative MAPK activating protein MP12	14706	5,1409	9	6	54

*C: coverage

Table 3. Proteins identified by MALDI-TOF in both tumor samples

Entry	Description	MW	pI	Score	Peptides	*C %
P10809	Heat shock protein 60	61016	5,5503	9	22	47
P30101	Disulfide isomerase ER 60	56746	5,9299	9	25	46
P02647	Apolipoprotein A I	30758	5,4309	8	15	47
P04792	Heat shock 27	22768	5,9588	9	10	55
P06727	A IV precursor	45371	5,1129	9	27	60
P16949	Stathmin	17292	5,76	6	29	76

*C: coverage

vealed important intracellular signalling modifications in tumor cells with activation of PI3K pathway. Experimental data may not be directly compared to the in vivo effects of radiotherapy on human patients due to higher complexity of histology in human MDB [18,19] and the sophisticated approach of patients radiotherapy treatment. Modifications of protein expression induced in vivo by radiotherapy alone on human MDB are practically unknown. The case presented was someway unique because the relapse occurred after radiotherapy alone without any contribution of chemotherapy. The radiotherapy induced differential protein expression showed a somewhat new scenario with three relevant aspects: 1) a reduced asset of proteins in MDB after radiotherapy, 2) the appearance of new proteins and 3) few conserved proteins. A better understanding of radiotherapy induced changes on MDB cells may offer the opportunity of a more rational selections of drug regimens or suggest innovative approaches

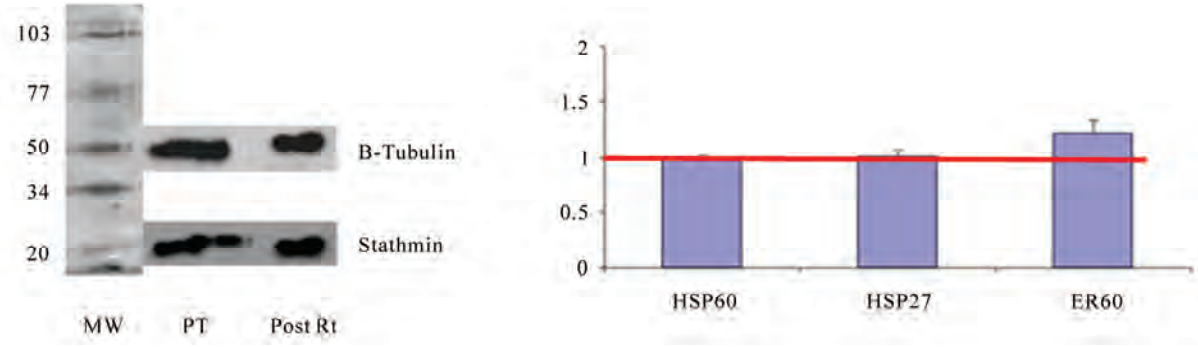

Figure 2. (a) Western blot analysis of primary tumor and post radiotherapy (Rt) stained by anti- STM1 and anti-β-Tubulin. (b) Real-Time PCR analysis of proteins expressed in both tumor in primary tumor and post radiotherapy. Results are present as fold increase in post radiotherapy vs primary tumor control. Data presented were normalized to S14 mRNA expression and are mean ± SD of three experiments

taking advantage of the reduced protein expression after radiotherapy [14].

Data presented confirmed the importance of Sthatmin as a marker of aggressiveness in Anaplastic MDB as this protein was one of the few proteins expressed in both of the tumor samples.

In fact the radiotherapy induced changes of protein expression, as evidenced by proteomic analysis, may represent an escape strategy of the tumor to survive to radiotherapy and a reversal to a more undifferentiated, stem cell-like status. On the other hand, the new protein expression patterns displayed by radiotherapy treated cells may modify cell sensibility to specific cytotoxic drugs or suggest innovative treatment strategies as the findings on the mouse model shows [17]. The expression of CD140a (PDGFRa) may be of particular interest as one of the possible target of anti-receptor small molecules of recent development [20].

4. Conclusions

Proteomic analysis of post "in vivo" radiotherapy of Anaplastic Medulloblastoma disclosed relavant modifications of protein profile. The reduction of proteins present in the relapse after radiotherapy alone was impressive: almost 40% of the total protein repertoire was lost and few "new" proteins were detected. The expression of CD 140 as a new radiotherapy induced protein could be of particular interest as a possible target of anti-receptor therapeutic approach. A proteomic guided chemotherapy based upon protein depauperation induced by radiotherapy could take advantage of the limited protein expression and target these proteins, precluding to the tumor any survival alternative.

A detailed study on a large number of cases may disclose new opportunities for combined therapy based on solid scientific data of radiotherapy effects and not only on long lasting empirical experience.

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TABLE OF CONTENTS

Volume 1 Number 2 **June 2010**

Research

Review

Case Report

