

Evaluation of Cytogenetic Alterations in Peripheral Blood Lymphocytes of Esophageal Cancer Patients Treated with Radiotherapy or Chemoradiotherapy using Cytokinesis-Blocked Micronucleus Assay

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Abstract- The effects of combined radiotherapy (RT) and chemotherapy in the severity of cytogenetic alterations expressed as micronucleus (MN) in peripheral blood lymphocytes of patients treated for esophageal cancer was evaluated. To do this, blood was obtained from 23 and 15 esophageal cancer patients scheduled for chemo-radiotherapy and RT alone, respectively, before, during, and after treatment. Blood samples were cultured in RPMI-1640 complete medium containing 1% phytohemagglutinin and incubated in a CO₂ incubator. Cytochalasin-B was added to the cultures at a final concentration of 5µg/ml. Finally, harvesting, slide making, and analysis were performed according to standard procedures. Results indicate that there was no significant difference between the frequencies of MN in lymphocytes of individuals before being treated with RT alone or chemo-radiotherapy. In the middle of treatment, (after 12 fractions of RT) the frequency of MN increased significantly compared with their concurrent pre-treatment samples in both groups (four-fold). However, the frequency of MN observed for RT patients was not significantly different with those received chemo- and radiotherapy. At the end of treatment, (after 24 fractions of radiotherapy) an increase in the MN frequency was observed for chemo-radiation group significantly higher than RT group ($P=0.022$). Mild increase in MN frequency in lymphocytes of patients receiving chemoradiation only after the completion of treatment course might be indicative of resistance induced by chemotherapeutics to the clastogenic effects of radiation. Therefore, using these agents repeatedly for cancer treatment in combination with radiation might not cause severe adverse biological effects in normal tissues.

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Introduction

Esophageal cancer is the eighth most common cancer worldwide, but it is the sixth most common cause of death from cancer, so esophageal carcinoma is a highly virulent disease (1). There are various treatment methods for esophageal cancer, such as surgery, chemotherapy, radiotherapy and different kinds of combined-modality therapies, but clinical outcome of esophageal cancer is poor. Surgery is the preferred and standard treatment for this cancer (2,3). For patients who

are not a candidate for surgical resection, combined-modality therapy (chemoradiation) has better results (2-4).

Several studies have indicated various advantages for chemoradiation treatment (combined chemotherapy and radiotherapy) as compared with radiotherapy or chemotherapy alone (5-10). Because combined chemotherapy drugs and radiation has additive and synergism effects in the killing of cancer cells (11,12), so treatment outcome will be better, but combined treatment also has disadvantages. It is more toxic to

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normal tissues as compared with other treatment methods (5,7,9,13,14), because its performance is not restricted to malignant cells. Radiotherapy and chemotherapy can cause DNA damage in normal cells, misrepaired, or unrepaired double-strand breaks in DNA lead to chromosomal breaks. As a result patients experience early and late effects in normal tissue during the therapy (15-17). However, there are experimental evidence showing the hormesis induced by chemotherapeutics might reduce the clastogenic effects of radiation in non-target tissues such as bone marrow (18,19).

Cytogenetic techniques can assess patient's complications during the therapy and used for detecting individuals (patient or healthy) with intrinsic sensitivity. Because there is an association between chromosome abnormalities and the risk of developing cancer (20) cytogenetic bioindicators can be used as a cancer predictive assay (21-26).

For evaluation of chromosome abnormalities in peripheral blood lymphocytes (PBL) of esophageal cancer patients, we used cytokinesis-block micronucleus (CBMN) assay. The CBMN assay is a mutagenic test system for detection of the formation of small membrane-bound DNA fragments (i.e. micronuclei in the cytoplasm of interphase cells) induced by chemical and physical agents. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are unable to migrate with the rest of the chromosomes during the anaphase of cell division (22,27,28,29). CBMN assay is a simple, reliable, sensitive, rapid and low-cost cytogenetic test (30,31).

The aim of the present study was to evaluate the possible side effects of combined radiotherapy and chemotherapy in peripheral blood lymphocytes of esophageal cancer patients. Several studies have assessed chromosome abnormalities and DNA damages induced by radiotherapy and chemotherapy alone in PBL of cancer patients (especially breast cancer) (11,16,22,27,28,30,32-39), but less attention has been made to the side effects of chemoradiation treatment of esophageal cancer patients.

Materials and Methods

Patients

Two groups of esophageal cancer patients were investigated. The first group consisted of 23 patients, including 12 men and 11 women from 42 to 89 years old (66.52 ± 12.075), scheduled for treatment with chemo-

radiotherapy. This group received chemotherapy drugs (cisplatin, 20 mg/m^2 and 5-FU, 300 mg/m^2) once a week 30 minutes to one hour before radiotherapy. Radiotherapy of these patients was done using a linear accelerator (Siemens, Primus, Multi-energy system) with a daily dose was 180 cGy at a dose rate of 200 cGy/min (five fractions per week). The second group comprised 15 patients, including 7 men and 8 women from 51 to 84 years old (70.07 ± 11.54) that were treated with radiotherapy alone with similar irradiation procedure described for the first group. The study was approved by the Ethics Committee of Urmia University of Medical Sciences, and patients gave their consent for blood donation and took part in the study voluntarily.

Blood sample collection

Blood sample (3-4 ml) from each patient was collected in heparinized vacutainers via venipuncture, during three stages of treatments; i.e., before initiation of chemotherapy and radiotherapy, during therapy after 12 fractions of RT (21.6 Gy) and finally at the end of treatment after 24 fractions of RT (43.2 Gy).

Lymphocyte culture for CBMN assay

Lymphocyte culture was initiated by addition of 0.5 ml of whole blood to 4.5 ml RPMI-1640 medium containing L-glutamine and 15% fetal bovine serum (FBS) (Gibco). The duplicate culture was set for each patient. Lymphocytes were stimulated with 1% phytohemagglutinin (PHA) (Gibco) and then samples were incubated in a CO₂ incubator at 37°C. Forty-four hours after the initiation of culture, cytochalasin-B (Sigma) was added to yield at a final concentration of 5 µg/ml. Twenty-eight hours later cells were harvested by centrifugation at 1000 rpm for 10 minutes. The cells were subjected to hypotonic treatment (0.075M, KCl) for 2min, centrifuged and fixed in fresh fixation solution (Methanol and Acetic acid 1:6 v/v). Fixation step was repeated twice after 20min at room temperature. After the last centrifugation, cells were re-suspended in a small volume of fixative and dropped onto pre-cleaned slides. Then slides were stained for 20min in 10% Giemsa solution.

One thousand binucleated lymphocyte cells were evaluated by light microscope (magnification 400×) for each sample, and the frequency of MN was recorded except for those samples obtained after the completion of chemoradiotherapy in which the number of assessed binuclei cells was less. The scoring criteria were those indicated by Fenech (29).

Results

obtained for each individual in the two groups are summarized in Table 1.

Patients' demography and detailed statistical data

Table 1. Alteration in the frequency of MN in peripheral blood lymphocytes of esophageal cancer patients in relation to treatment modality and received radiation dose.

Patient ID no.	Age	Sex	Before treatment			After 12 irradiation fraction			After 24 irradiation fraction		
			BN	MN frequency	MN percentage	BN*	MN frequency	MN percentage	BN	MN frequency	MN percentage
Radiotherapy alone											
01	84	M	1000	53	5.3%	1000	347	34.7 %	423	237	56.03 %
02	84	M	1000	72	7.2 %	554	147	26.53 %	500	175	35%
03	71	M	1000	73	7.3 %	1000	209	20.9 %	1000	263	26.3 %
04	70	F	1000	40	4 %	800	191	23.87 %	758	225	29.68 %
05	58	M	1000	74	7.4 %	700	224	32 %	500	175	35%
06	74	F	1000	102	10.2 %	923	244	26.44 %	142	45	31.69 %
07	58	F	1000	69	6.9 %	1000	213	21.3 %	1000	295	29.5 %
08	61	M	502	23	4.58 %	241	44	18.26 %	300	105	35%
09	51	F	1000	39	3.9 %	1000	201	20.1 %	1000	225	22.5 %
010	81	M	1000	32	3.2 %	1000	289	28.9 %	1000	417	41.7 %
011	71	F	1000	33	3.3 %	1000	250	25 %	1000	327	32.7 %
012	66	M	1000	25	2.5 %	1000	247	24.7 %	1000	332	33.2 %
013	84	F	1000	43	4.3 %	1000	300	30 %	1000	527	52.7 %
014	83	F	1000	27	2.7 %	663	269	40.57 %	486	163	33.5 %
015	55	F	1000	69	6.9 %	291	64	21.99 %	136	42	30.88 %
Mean(%)±S.D	70.07±11.55				5.31%±2.22			26.35%±6.05			35.03%±8.98
Chemoradiation											
1	52	F	1000	37	3.7 %	901	243	26.96%	1000	406	40.6 %
2	67	M	800	42	5.25 %	1000	230	23 %	326	140	42.94 %
3	60	M	1000	56	5.6 %	1000	435	43.5 %	1000	666	66.6 %
4	63	M	1000	32	3.2 %	1000	214	21.4 %	1000	311	31.1 %
5	69	F	1000	77	7.7 %	1000	198	19.8 %	1000	500	50 %
6	42	M	1000	66	6.6 %	1000	182	18.2 %	1000	316	31.6 %
7	82	M	1000	54	5.4 %	900	192	21.33 %	365	92	25.2 %
8	75	M	1000	65	6.5 %	1000	203	20.3 %	150	67	44.66 %
9	68	M	1000	97	9.7 %	1000	188	18.8 %	600	221	36.83 %
10	67	F	1000	82	8.2 %	927	168	18.12 %	1000	358	35.8 %
11	46	M	1000	77	7.7 %	1000	315	31.5 %	241	138	57.26 %
12	55	F	1000	59	5.9 %	901	243	26.96%	665	204	30.67 %
13	71	M	1000	54	5.4 %	1000	256	25.6 %	1000	428	42.8%
14	68	F	1000	59	5.9 %	1000	256	25.6 %	559	210	37.57 %
15	89	M	860	54	6.3 %	674	196	29 %	364	165	45.33 %
16	76	M	1000	49	4.9 %	1000	212	21.2 %	324	59	18.2 %
17	56	F	1000	57	5.7 %	1000	438	43.8 %	231	144	62.34 %
18	78	F	1000	74	7.4 %	467	96	20.56 %	1000	574	57.4 %
19	80	F	604	37	6.13%	208	64	30.77 %	216	87	40.27 %
20	52	F	1000	68	6.8 %	864	228	26.38 %	415	297	71.56 %
21	63	M	864	53	6.13%	1000	289	28.9 %	669	259	38.71 %
22	80	F	1000	77	7.7 %	437	141	32.26 %	1000	348	34.8 %
23	71	F	1000	32	3.2 %	593	273	46.04 %	500	214	42.8%
Mean(%)±S.D	66.52±12.07				6.13%±1.56			26.95%±8.16			42.83%±13.12

In the chemoradiation group, the mean spontaneous frequency of micronuclei in the pre-treatment stage (0Gy) was 61.3 per one thousand binucleate lymphocyte cells (6.13% ± 1.56). In the radiotherapy alone, group the average frequency of micronuclei before the treatment was 53.1 per one thousand binucleate

lymphocyte cells (5.31% ± 2.22). In both groups, there were inter-individual differences in the spontaneous frequency of micronuclei.

In the second stage, after 12 fractions (21.6Gy) the frequency of micronuclei increased significantly compared with their concurrent pre-treatment samples in

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both groups (four-fold or more). After 12 fractions of treatment, the average frequency of micronuclei in lymphocytes was $26.95\% \pm 8.16$ and $26.35\% \pm 6.05$ in chemoradiation and radiotherapy alone groups, respectively. In comparison with radiotherapy alone treatment, chemoradiation group had a higher frequency of micronuclei, but the difference was not statistically significant ($P= 0.929$).

The assessment of blood samples collected after 24 fractions (43.2Gy) confirmed an increase in the micronuclei frequency proportional to increasing radiation doses. In this stage, the mean frequency of micronuclei was $42.83\% \pm 13.12$ and $35.03\% \pm 8.98$ in chemoradiation and radiotherapy alone groups respectively, the difference was statistically significant ($P= 0.022$) (Figure 1).

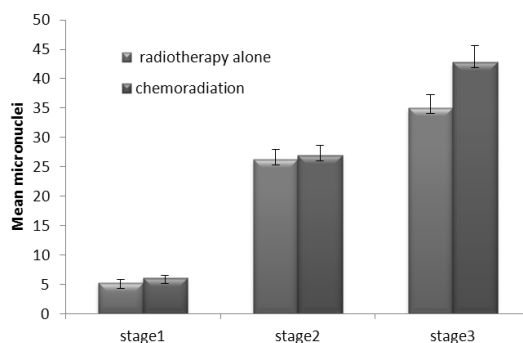


Figure 1. Comparison of mean MN in radiotherapy alone and chemoradiation groups in three stages

Some binucleate lymphocytes in the pre-treatment samples of cancer patients had two micronuclei (MN). However, subjects in the middle of therapy showed an increase in the rate of 2MN and 3MN in the binuclei cells and a few samples had binucleates with 5-7MN. A similar observation was made in the post-treatment samples.

In the chemoradiation group, age had no effect on the frequency of micronuclei, but in the radiotherapy alone group age affected the MN frequency mostly in the second and third stages, but had no effect in the first stage. Also, no gender effect was observed on the frequency of micronuclei in both groups.

Discussion

Radiotherapy and chemotherapy can cause DNA damage in normal cells not targeted for treatment, misrepaired, or unrepaired double-strand breaks (DSB) in DNA lead to chromosomal breaks. These cytogenetic alterations in cells lead to cell death and improve the efficacy of radiotherapy or chemotherapy of tumors. However, there are also adverse side effects associated with each treatment modality especially radiosensitive tissues in bone marrow and circulating blood. These side effects often prevent the oncologists from delivering appropriate doses of drugs or radiation for better curability. As seen in Table 1 and Figure 1, high frequency of micronuclei was observed in lymphocytes

of esophageal cancer patients before any treatment. Several studies have shown that DNA damage and chromosome abnormalities in PBL of cancer patients are higher than the healthy control group (11,16,22,27,28,31,35,38), indicating higher genome instability of cancer patients.

In the present study, the mean frequency of spontaneous micronuclei didn't differ in both groups of cancer patient samples collected before the treatment. The analysis of micronuclei in both groups showed a wide inter-individual variation between patients. This finding agrees with other studies (16,17,28,33), which show inheritance differences and different radiosensitivity among individuals.

Samples that were collected in the middle of treatment in both groups showed a significant increasing in the micronuclei frequency as compared with their concurrent pre-treatment samples in both groups. This increase was four-fold, and for some patients was greater than fourteen-fold, that it is due to radiation effects. This increasing in the micronuclei frequency agrees with other investigations (28,30,36). Moreover, we found that chemoradiation group had a higher frequency of micronuclei than radiotherapy alone group (but the difference was not statistically significant). The assessment of blood samples collected at the end of treatment in both groups confirmed a significant elevation in the micronuclei frequency compared with their concurrent pre-treatment as well as mid-treatment

samples. Samples showed a six-fold or more increase in the micronuclei frequency. This elevation was proportional to increasing radiation doses. This finding is in agreement with other reported studies (28,30,36). Also, in this stage, the frequency of micronuclei in the chemoradiation group was significantly higher than radiotherapy alone. This result might be due to the accumulation of damages induced in the course of combined radiotherapy and chemotherapy. However, this increase was not so appreciable compared to those received radiotherapy alone. Previously, Mozdarani *et al.*, (18,19) in experiments with laboratory animals have shown that chemotherapy with actinomycin D and bleomycin sulfate before the whole body or fractionated irradiation cause resistance or adaptation of bone marrow cells to radiation. Wolff *et al.*, showed no significant increase in the frequency of micronuclei after chemotherapy in lymphocytes (30). Therefore, this mild effect of chemotherapeutics on lymphocytes might have triggered their repair machinery to tolerate higher doses of radiation.

Regarding the age and gender effect on MN formation, although there was generally no age and gender effect seen in the current study except in some patients receiving radiotherapy alone, there is no general agreement of the age or gender effect of MN formation. Some studies have reported the effect of age and gender on the frequency of micronuclei in the PBL of patients (27,41,42). However, other studies have showed no age and sex effect on the frequency of micronuclei (22,28,43-45).

In conclusion, high frequency of micronuclei in normal lymphocytes was seen after completion of the treatment course but not much higher than those induced by radiation alone.

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