A study on the best irradiation dose of X-ray for Hep-2 cells by Fourier transform infrared spectroscopy

Renming Liu (刘仁明)[1], Weiyue Tang (唐伟跃)[2], Guangshui Zhang (张广水)[2], Fengqiu Zhang (张凤秋)[2], and Xinhui Yan (阎新惠)[3]

[1]*Department of Physics and Electronic Science, Chuxiong Normal University, Chuxiong 675000*

[2]*Physical Engineering College, Zhengzhou University, Zhengzhou 450052*

[3]*The People's Hospital of Henan Province, Zhengzhou 450000*

Received November 26, 2007

Fourier transform infrared spectroscopy (FTIR) was employed to study the human epidermis larynx carcinoma cell lines (Hep-2) which were irradiated by different doses of X-ray. The results show that (1) the irradiation of X-ray damages the structure of the CH_3 groups of the thymine in DNA, which restrains the reproduction of Hep-2 cells effectively, (2) the 8 Gy dose of X-ray irradiation changes the framework and the relative contents of some proteins, lipids and the nucleic acid molecules intercellular in the greatest degree, and (3) the 8 Gy dose of X-ray irradiation is the best irradiation dose for lowering the degree of the cancerization of Hep-2 cells according to the criteria for the degree of the cancerization reported recently. Meanwhile, the apoptosis of these cells were detected by using flow cytometry (FCM) primarily. It shows that the apoptotic ratio of the Hep-2 cells depends on the irradiation dose to some extent, but is not linearly. And the apoptotic ratio of the 12 Gy dose group is the maximum (20.36%), but the apoptotic ratios of the 2 to 8 Gy dose groups change little.

OCIS codes: 300.6300, 040.3060, 340.7480, 170.1530.

Larynx cancer is a kind of familiar carcinomas of the head neck cancers, and the incidence of this cancer account for about 5% of all human cancers worldwide. Unfortunately, the incidence of this cancer is rising recent years with the air pollution, smoking, drinking, and other factors. Especially, smoking is one of the most principal inducements to this cancer. The ways of the therapy for the larynx carcinomas are chemotherapeutics, actinotheraphy, and operations. As we all known that X-ray is a kind of short wave electromagnetic waves, when the carcinomatous cells are irradiated by X-ray, some proteins, lipids, and the nucleic acid will be damaged or repaired synchronously, so X-ray radiation would induce the startup of some apoptotic mechanism[1−4]. Consequently, X-ray has been used to cure the malignant tumors in clinic up to today. But to the clinic, there is not a very scientific dose reference of X-ray irradiation for the larynx cancer therapy, and the design of the irradiation dose is always according to the experiences of the doctors, so it is quite subjective. Spectroscopic analysis is an effective method in analyzing structure, components, and the contents of various materials due to its superduper sensitivity[5−8]. It is one of the most effective tools for studying the stages of the biomolecules and cells. Infrared radiation (IR) has become one of the most common tools in analyzing since its discovery in 1801 by Willism Herschel. And with the development of the computer technology and the data processing technology in the mid-1970s, Fourier transform infrared spectroscopy (FTIR) has developed to be one of the most important analytic tools in many fields. Recently, FTIR was employed to study the cells or tissues of the uterus, colon, liver, lung, breast, and other cancers[9−11]. The merits of it are accurate, convenient, and economical. Thus we employ the FTIR technique to study the best X-ray irradiation dose for Hep-2 cells in this paper, and expect to provide a reference of X-ray irradiation dose for the human larynx carcinomas therapy in the future.

A human laryngeal squamous cell carcinoma cell line (Hep-2) was obtained from the pathology lab of Zhengzhou University, China. The cell line was maintained in RPMI1640 medium (Gibco Co., USA), supplemented with 10% heat-inactivated fetal bovine serum (Gibco Co.) at 37 °C in a humidified 5% CO_2 atmosphere. The cells were harvested in exponential growth phase from the culture medium, counted and replanted in 35 culture dishes (the number of the cells in each culture dish is the same), labeled and cultured for 48 h subsequently.

These 35 culture dishes were divided into 7 groups (each group included 5 parallel groups) which included 6 different X-ray irradiation dose groups (1, 2, 5, 8, 10 and 12 Gy) and a control group (0 Gy) for X-ray irradiation. The X-ray irradiation was carried out at room temperature by using an X-ray generator (6MeV-linear accelerator, Siemens Co., Germany), the distance between the orifice which X-ray came out from and the culture dishes was 100 cm, and the irradiation area was 10×10 (cm). After that, these cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere for 48 h sequentially. Then, taking out all the 35 culture dishes of the cells to harvest, count, and wash in phosphate buffer saline (PBS) 3 times gently (each group had a same cell number of 10^6). Lastly, a vacuum freeze-drying equipment (ALPHA 1-2LD, CHRIST Co., Germany) was employed to dry the cells and then the FTIR spectra in the range of $4000 - 500$ cm^{-1} were collected by Fourier transform infrared spectrometer (NEXUS-470, Nicolet Co., USA), as shown in Fig. 1. Table 1 shows the absorption bands in FTIR spectra and their assignation. And the intensity ratios at 1654/1542, 1454/1400, and 2958/2847 cm^{-1} for each group are shown in Fig. 2 and Table 2. On the other

1671-7694/2008/050369-04

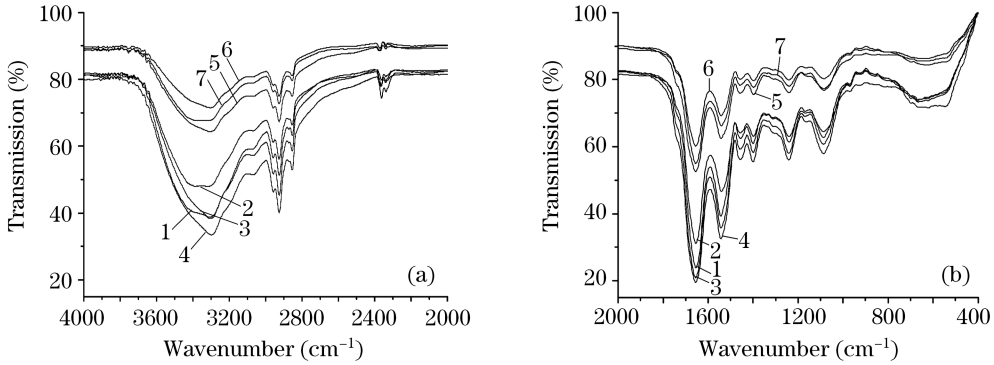

Fig. 1. FTIR spectra of the Hep-2 cells irradiated by X-ray and cultivated for 48 h sequentially. (a) Spectra in the range of 4000 − 2000 cm^{-1}; (b) spectra in the range of 2000 − 400 cm^{-1}. 1: 0, 2: 1, 3: 2, 4: 5, 5: 8, 6: 10, 7: 12 Gy dose group, respectively.

Table 1. Average Values of the Bands ($\bar{x} \pm s$) and Their Assignations in FTIR Spectra

C_1	C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}	
Control	3314.48	2958.7	2925.00	2847.53	1654.60	1542.86	1454.38	1400.38	1239.59	1086.68
Group	±1.04	±1.33	±0.76	±1.28	±0.98	±1.65	±1.42	±1.36	±0.64	±1.30
1 Gy	3310.84	2958.73	2925.7	2851.86	1654.72	1540.62	1457.23	1398.53	1239.67	1686.75
	±0.28	±1.33	1 ± 0.65	±0.99	±1.23	±1.73	±0.59	±1.30	±0.82	±1.04
2 Gy	3304.9	2958.87	2925.43	2854.65	1655.52	1542.24	1456.76	1399.38	1240.20	1086.60
	7 ± 0.89	±1.03	±0.64	±1.42	±1.38	±1.51	±1.01	±1.37	±1.35	±0.68
5 Gy	3298.64	2958.26	2925.49	2854.77	1655.21	1542.70	1455.82	1400.42	1240.00	1086.49
	±1.53	±1.63	±0.76	±1.04	±1.30	±1.57	±1.53	±1.29	±0.93	±1.20
8 Gy	3307.58	2958.35	2525.69	2855.94	1655.66	1542.37	1457.26	1397.38	1239.91	1084.78
	±0.93	±0.91	±0.82	±1.14	±0.80	±0.90	±1.01	±1.22	±0.97	±1.08
10 Gy	3306.22	2958.06	2926.25	2855.88	1656.16	1541.20	1457.14	1396.60	1240.11	1084.57
	±0.58	±1.27	±0.48	±0.94	±0.39	±1.30	±0.94	±1.33	±0.98	±0.93
12 Gy	3309.70	2959.42	2926.33	2851.62	1655.20	1541.24	1457.16	1397.96	1239.47	1084.92
	±0.92	±1.21	±0.60	±1.24	±1.57	±1.16	±0.77	±1.13	±0.48	±1.17
Assignation	ν (N-H)	$\nu_{as}(CH_3)$	$\nu_{as}(CH_2)$	$\nu_s(CH_2)$	Amide I α-Helix	Amide II α-Helix	$\delta(CH_2)$	$\delta_s(CH_3)$	$\nu_{as}(PO_2^-)$	$\nu_s(PO_2^-)$

C: average values of the bands (cm^{-1}), ν: stretching vibration, ν_s: symmetric stretching vibration, ν_{as}: asymmetric stretching vibration; δ: bending vibration, δ_s: symmetric bending vibration.

Table 2. Average Values ($\bar{x} \pm s$) of the Intensity Ratios at 1654/1542, 1454/1400, and 2958/2847 cm^{-1} for Each Groups

	0 Gy	1 Gy	2 Gy	5 Gy	8 Gy	10 Gy	12 Gy
I_{1654}/I_{1542}	1.252	1.292	1.232	1.198	1.184	1.291	1.333
	±0.004	±0.012	±0.010	±0.007	±0.004	±0.006	±0.004
I_{1454}/I_{1400}	0.960	0.972	0.976	0.983	0.993	0.975	0.971
	±0.018	±0.028	±0.012	±0.022	±0.018	±0.019	±0.010
I_{2958}/I_{2847}	1.176	1.157	1.167	1.149	1.138	1.148	1.163
	±0.018	±0.002	±0.010	±0.006	±0.005	±0.007	±0.003

hand, the apoptotic ratios of the cells were detected by using flow cytometry (FCM) (FACS Vantage SE, BD Co., USA), and the cells were stained with Annexin V-FITC/PI (Sigma Chemical Co., USA). Figure 3 shows the apoptotic results of Hep-2 cells.

The bands at around 1654 and 1542 cm^{-1} are attributed to Amide I and Amide II. According to some correlative studies, the ratio of the peaks intensity I_{1654}/I_{1542} is an effective criterion for the degree of the tissue's cancerization[12], and the value of the ratio is always lower in normal tissues than that of in the malignant tissues. So we study the ratio for different irradiation dose groups and find that the value of the ratio of the 8 Gy dose group is the lowest (Table 2 and Fig. 2(a)),

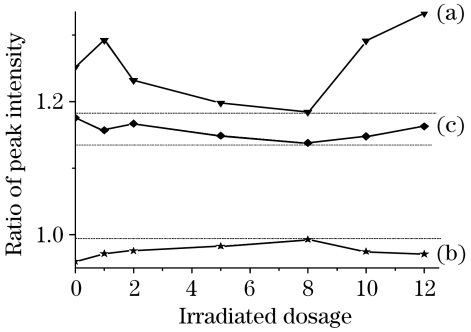

Fig. 2. Varieties of the intensity ratios at 1654/1542, 1454/1400, and 2958/2847 cm^{-1} for each group with the dose of X-ray irradiation. (a) I_{1654}/I_{1542}; (b) I_{1454}/I_{1400}; (c) I_{2958}/I_{2847}.

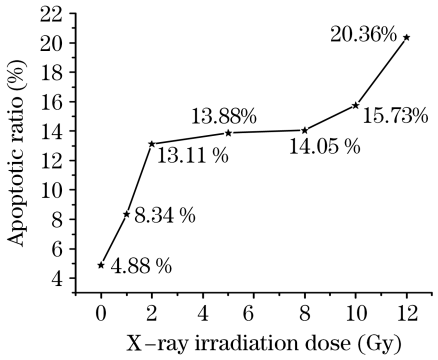

Fig. 3. Apoptotic ratios of Hep-2 cells of the different dose groups (0, 1, 2, 5, 8, 10, 12 Gy).

suggesting that the cancerization degree of the cells of this group is the lowest. It is possible that the 8 Gy irradiation of X-ray changes the relative contents of Amide I and Amide II greatly and restrains the normal metabolism of Hep-2 cells to a great extent, so the degree of the cancerization of the cells is reduced maximally.

The weaker aminoacid side chain from peptides and proteins of backbone DNA at around 1454 cm^{-1} is associated with the asymmetric CH_3 bending vibrations ($\delta_{as}CH_3$)[7,13]. And the decrease in intensity of this band may indicate specific conformational change of backbone DNA following its interaction with nuclear proteins such as transcription factors[7]. Compared with the control group, this band at around 1454 cm^{-1} of the different dose groups has a blueshift about $1-3$ cm^{-1}. It is related to the electronegative groups or the unsaturated groups connecting to the CH_3 group, which changes the conformation of backbone DNA. The band at around 1400 cm^{-1} is attributed to the CH_3 symmetry bending vibration of the thymine in DNA. Compared with the control group, this band of the different dose groups has a redshift about $1-3$ cm^{-1}, suggesting that the irradiation of X-ray damages the framework of the thymine of DNA, accordingly, restrains the replicating of DNA and makes the cells proliferation become slower. From the FTIR spectra (Fig. 1(b)), we can see that the absorption intensity of this band become weaker compared with that of the control group, suggesting that the relative contents of DNA decrease, i.e. the replication of DNA become weaker. Xu *et al.*[14] pointed out that the ratio of the peaks intensity at 1454 and 1400 cm^{-1} was also an effective criterion for the degree of the cancerization. The value of the ratio of the normal tissues is $I_{1454}/I_{1400} > 1$, and that of the malignant tissues is $I_{1454}/I_{1400} < 1$. On the other hand, the closer the value of the ratio approaches to 1, the lower the cancerization of the tissues will be. From Table 2 and Fig. 2, it is obvious that the maximum value of the ratio is the 8 Gy dose group and which approaches close to 1, suggesting that the cancerization degree of the cells of the 8 Gy dose group is the lowest, which accords with the conclusion above.

The 1086-cm^{-1} band is attributed to the symmetry stretching mode of the phosphodiester group ($\nu_s PO^{2-}$). Compared with the lower dose group, the band of the higher dose groups (8, 10, and 12 Gy) has a redshift about 2 cm^{-1}, it shows that the hydrogen banding of the PO^{2-} group is enhanced[15]. As to the asymmetric stretching mode of the phosphodiester group ($\nu_{as}PO^{2-}$) at around 1239 cm^{-1} in FTIR spectra, the frequency shift of which is not obvious. But from Fig. 1(b), it is found that the absorption intensities of the two bands for the higher dose groups (8, 10, and 12 Gy) become weak greatly, which indicates that the relative contents of the nucleic acid intracellular for these dose groups decrease greatly, i.e. the relative contents of DNA of the cells for these groups decrease, namely, the replicating of DNA is restrained greatly, because the intensities of the two bands indicate the content of the nucleic acid of DNA intracellular.

The bands at around 2958, 2925, and 2847 cm^{-1} in FTIR spectra (Fig. 1(a)) are responsible for the asymmetric stretching vibration of the CH_3 group ($\nu_{as}CH_3$), the asymmetric and symmetric stretching vibration of the CH_2 group of the lipid molecules, respectively. Compared with the control group, the bands of the different dose groups at 2958 and 2925 cm^{-1} do not shift almost, but the band near 2847 cm^{-1} has a blueshift about $4-8$ cm^{-1}, especially for the 8 and 10 Gy dose groups, which shifts upwards approaching to 8 cm^{-1}. The stretching mode of the CH_2 group (νCH_2) implies the inordinance degree of the hydrocarbon chain of the lipid molecules intracellular[16]. It interprets that the inordinance degree of the CH_2 chain in membrane lipid of the cells of the 8 Gy dose group is the largest. From Fig. 2(c), we can see that the value of the ratio I_{2958}/I_{2847} of the 8 Gy dose group is the minimum, implying that the relative contents of CH_3 and CH_2 groups in lipid molecule are changed maximally at the 8 Gy dose of X-ray irradiation.

On the other hand, we studied the apoptotic ratio of the Hep-2 cells which were irradiated by different dose of X-ray and cultivated for 48 h sequentially by using FCM, and the cells were stained with Annexin V-FITC/PI. The experimental results show that the apoptotic ratio depends on the irradiation to some extent, but is not linearly (Fig. 3). It is found that the apoptotic ratio of the 12 Gy dose group is the maximum (20.36%), but the apoptotic ratios of the 2 to 8 Gy dose groups change little and it is almost at an apoptotic platform. According to the clinic, the therapy dose of X-ray irradiation for the larynx cancer is commonly about $60-70$ Gy within $6-7$ weeks (accumulatively), and has a weaker injure to the normal tissues. Combining the FTIR analysis above, we find that the 8 Gy is the best dose of X-ray for lowering the degree of the cancerization of Hep-2 cells in the irra-

diation dose groups which we designed.

In conclusion, the FTIR spectra of Hep-2 cells, which were irradiated by the different dose of X-ray and cultivated for 48 h sequentially, were obtained by FTIR spectrometer. It is shown that, after Hep-2 cells irradiated by the different dose of X-ray, the structure and the relative contents of proteins, lipids, and the nucleic acid intracellular change obviously. From the analysis, there are some shifts in different degree of the absorption bands near 1454, 1086, and 2847 cm^{-1}, which indicates that the X-ray irradiation has some effect on conformation and relative contents of some biomolecules of Hep-2 cells. According to some criteria of the cancerization degree, it is quite obvious that the cancerization degree of the Hep-2 cells of the 8 Gy dose group is the lowest. And with the FCM analysis, we can see that the apoptotic ratio of the 12 Gy dose group is the maximum (20.36%), but the apoptotic ratios of the 2 to 8 Gy dose groups change little. According to the clinic and our analysis, we conclude preliminarily that the 8 Gy of the X-ray irradiation is the best X-ray irradiation dose for lowering the cancerization degree of Hep-2 cells.

This work was supported by the Science and Technology Department of Henan province (No. 0624410052), the key Ion Beam Bioengineering Laboratory of Henan Province, the Pathology Laboratory of Zhengzhou University, and the People's Hospital of Henan Province. R. liu's e-mail address is liurenmingok@126.com.

References

1. K. Ohnishi, I. Ota, A. Takahashi, K. Yane, H. Matsumoto, and T. Ohnishi, Apoptosis **7,** 367 (2002).
2. K. Iwamoto, N. Shinomiya, and H. Mochizuki, Apoptosis **4,** 59 (1999).
3. Z.-G. Cui, T. Kondo, L. B. Feril, Jr., K. Waki, O. Inanami, and M. Kuwabara, Apoptosis **9,** 757 (2004).
4. M. Radojcic and N. E. A. Crompton, Radiat. Environ. Biophys. **40,** 131 (2001).
5. Y. Li, R. Chen, H. Zeng, Z. Huang, S. Feng, and S. Xie, Chin. Opt. Lett. **5,** 105 (2007).
6. Y. Shi, G. Liu, J. Liu, and D. Song, Acta Opt. Sin. (in Chinese) **27,** 129 (2007).
7. X. Gao, I. S. Butler, and R. Kremer, Spectrochimica Acta Part A **61,** 27 (2005).
8. L. Tang, Z. Sun, J. Guo, and Z. Wang, Chin. Opt. Lett. **2,** 101 (2006).
9. P. T. T. Wong, R. K. Wong, T. A. Caputo, T. A. Godwin, and B. Rigas, Proc. Natl. Acad. Sci. USA **88,** 10988 (1991).
10. C. M. Krishna, G. D. Sockalingum, R. A. Bhat, L. Venteo, P. Kushtagi, M. Pluot, and M. Manfait, Anal Bioanal Chem. **387,** 1649 (2007).
11. Y. K. Min, T. Yamato, E. Kahada, T. Ito, and H. Hamaguchi, J. Raman Spectrosc. **36,** 73 (2005).
12. J. Wang, Y. Xu, J. Shi, L. Zhang, X. Duan, L. Yang, Y. Su, S. Weng, D. Xu, and J. Wu, Spectrosc. Spectr. Analy. (in Chinese) **23,** 863 (2003).
13. J. Ramesh, A. Salman, S. Mordechai, S. Argov, J. Goldstein, I. Sinelnikov, S. Walfisch, and H. Guterman, Subsurf. Sens. Technol. Appl. **2,** 99 (2001).
14. Y. Xu, Y. Zhao, Z. Xu, Y. Ren, Y. Liu, Y. Zhang, X. Zhou, J. Shi, D. Xu, and J. Wu, Spectros. Spectr. Analy. (in Chinese) **25,** 1775 (2005).
15. H. Huo, X. Hu, H. Guan, X. Wang, X. Che, and W. Huang, Chem. J. Chin. Univ. (in Chinese) **21,** 1244 (2000).
16. F. Wang, X. Ling, Z. Yang, Z. Xu, Y. Ren, W. Li, S. Weng, and J. Wu, Spectros. Spectr. Analy. (in Chinese) **23,** 498 (2003).