Saudi Journal of Biological Sciences (2016) **23**, 426–433

King Saud University

Saudi Journal of Biological Sciences

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ORIGINAL ARTICLE

Effect of long-term exposure to mobile phone radiation on alpha-Int1 gene sequence of *Candida albicans*

Ariyo Shahin-jafari [a], Mansour Bayat [a], Mohammad Hassan Shahhosseiny [b,*], Parviz Tajik [a], Shahla Roudbar-mohammadi [c]

[a] *Department of Pathobiology, Faculty of Veterinary Specialized Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran*
[b] *Department of Microbiology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran*
[c] *Faculty of Medical Mycology, Tarbiat Modares University, Tehran, Iran*

Received 1 March 2015; revised 11 April 2015; accepted 2 May 2015
Available online 9 May 2015

KEYWORDS

Mutagenesis;
Alpha-Int1 protein;
Microwaves;
Candida albicans;
PCR

Abstract Over the last decade, communication industries have witnessed a tremendous expansion, while, the biological effects of electromagnetic waves have not been fully elucidated. Current study aimed at evaluating the mutagenic effect of long-term exposure to 900-MHz radiation on alpha-Int1 gene sequences of *Candida albicans*. A standard 900 MHz radiation generator was used for radiation. 10 ml volumes from a stock suspension of *C. albicans* were transferred into 10 polystyrene tubes. Five tubes were exposed at 4 °C to a fixed magnitude of radiation with different time periods of 10, 70, 210, 350 and 490 h. The other 5 tubes were kept far enough from radiation. The samples underwent genomic DNA extraction. PCR amplification of alpha-Int1 gene sequence was done using one set of primers. PCR products were resolved using agarose gel electrophoresis and the nucleotide sequences were determined. All samples showed a clear electrophoretic band around 441 bp and further sequencing revealed the amplified DNA segments are related to alpha-Int1 gene of the yeast. No mutations in the gene were seen in radiation exposed samples. Long-term exposure

* Corresponding author at: Department of Microbiology, Shahr-e-Qods Branch, Islamic Azad University, Tehran, Iran. Tel./fax: +98 2188665120.
E-mail addresses: shahhosseiny@yahoo.com, mars_venus1978@ yahoo.com (M.H. Shahhosseiny).
Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

http://dx.doi.org/10.1016/j.sjbs.2015.05.001

of the yeast to mobile phone radiation under the above mentioned conditions had no mutagenic effect on alpha-Int1 gene sequence.

1. Introduction

Electromagnetic waves and related fields radiating from mobile phones usually influence many biological functions, and have extensively been investigated by many scientists (Adey, 1993; Levallois et al., 1999; Mahrour et al., 2005). Mobile phones radiate at frequencies of 900–1800 MHz and the radiation itself belongs to a wide range of microwave radiations in frequencies ranging from 300 MHz to 300 GHz. In turn, microwaves itself belong to a broader spectrum of electromagnetic waves in frequencies ranging from 10^0 Hz to more than 10^{20} Hz. Radio waves, with the longest wavelength of about 10^3 m, are located at one end of the EM spectrum, while gamma-rays with the shortest wavelength of about 10^{-12} m are located at the other end of the spectrum. The relationship between frequency and wavelength is reciprocal which means when the frequency rises the wavelength falls and vice versa. All radiations usually carry energy. The shortest waves, like gamma and X-rays, radiate as high energy photons that upon striking substances can easily break chemical bonds between atoms and exert an ionizing effect. On the other hand, the photons of radio waves, EM with the longest wavelength, carry low levels of energy and, therefore, are not able to break chemical bonds and to ionize atoms. Longwave radiations, including a microwave portion of the EM spectrum, generally have thermal effects and upon impact lead to a temperature rise in substances (true for both live and dead substances). The waves also show non-thermal effects on living cells and tissues. Regulatory bodies on many countries usually set safety standards for cell phone radiation according to the thermal effects of the waves on living systems (Dardalhon et al., 1985). Microwaves are radiated from radar equipment's, telecommunication and data transferring tools, radio stations, satellites, mobile cellphones etc. The radiations are further increased in the last decade due to the rapid progression and the tremendous expansion of technologies.

Iran's telecommunication network, like in many other countries, is based on the Global System for Mobile communication (GSM) standards and applies to frequency ranges of 900–1800 MHz for data transferring. For decades, intensive academic research has been done in industrialized countries to visualize biological impacts of mobile phone radiation, and in Iran, as a developing country, several valuable investigations have been done in the field in which the efforts of Dr. Seyed Mohammad Pourmir Firoozabadi are notable (Jadidi et al., 2007; Mansourian et al., 2012).

The main objective of the present study was, exploring the biological impacts of mobile phone radiation on common fungi, a normal resident of the human skin and mucus membranes, *Candida albicans*. To the best of our knowledge this is an innovative approach considering the used live system and the type of exploration. On the other hand, abundant occurrences of yeast in the skin and mucus membranes of humans, which are in close contact with mobile phone radiation, are re-duplicated in the significance of the investigation.

2. Materials and methods

2.1. Strains and radiation generator

C. albicans with ATCC number of 10231 was used through all stages of the study. A single colony from stock culture (slant agar) of *C. albicans* was transferred into the GYEP (glucose-yeast extract-peptone) broth medium and incubated at 37 °C for 48 h. *Fusarium solani* (ATCC: 36031), *Fusarium oxysporum* (ATCC: 48112), *Cryptococcus neoformans* (ATCC: 32045), *Saccharomyces cerevisiae* (ATCC: 9763), *Aspergillus flavus* (ATCC: 200026), *Aspergillus parasiticus* (ATCC: 15517) and *Aspergillus niger* (ATCC: 1015) strains were used for the determination of assay specificity. Mobile phone radiation simulator was a jammer type device with omnidirectional antenna and a maximum output power of 900 mW. The device is capable of producing microwaves at the two frequency ranges of 851–960 and 1805–1990 MHz and the first frequency range, 960–851 MHz, was used for the study. This device can tolerate 30–95% humidity and a temperature range of 40–55 °C.

2.2. Study design for C. albicans exposure to 900 MHz radiation

The aim of the current study was to experimentally evaluate the mutagenic effects of long term exposure of *C. albicans* to mobile phone radiation on nucleotide variation of alpha-Int1 gene of the yeast. *C. albicans* from the exponential phase of the yeast grown on GYEP broth medium was used for all experiments. A total number of 20 million cells of *C. albicans* (10 ml for each tube), suspended in GYEP medium, were transferred into five polystyrene tubes and exposed with proposed study design to radiation generator while incubated at 4 °C. The tubes were in close contact with the generator (maximum distance was 15 cm) and different exposure times of 10, 70, 210, 350 and 490 h were applied. According to the manufacturer recommendation, maximum time period of 3 h radiation was allowed, though 10 h per day exposure time was divided into three courses of 3.5 h with one hour resting intervals for considering safety issues of the device. A second series of tubes containing the same amount of candida cells (as control) were incubated at 4 °C far from the radiation. At each time point of 10, 70, 210, 350 and 490 h, one tube from exposed and another tube from unexposed groups were selected for DNA extraction. DNG-Plus DNA extraction kit from SinaClon (Cat. No.: DN8118C) was used for *C. albicans* genomic DNA extraction.

2.3. DNA extraction

For *C. albicans* genomic DNA extraction of 1 ml from each tube containing well suspended cells was transferred into a 1.5 ml Eppendorf tube and centrifuged at 8000 rpm for 5 min for removal of GYEP medium and to pellet yeast cells. The supernatant was discarded and the pellet was resuspended in

100 µl of DW and well mixed with 400 µl DNG-plus extraction buffer. 350 µl of isopropyl alcohol was then added to the mixture and vortexed. The mixture was centrifuged at 12,000 rpm for 10 min and decanted by gently inverting the tube and using tissue paper. One ml of 70% ethanol was then added and vortexed. The tube was centrifuged again at 12,000 rpm for 8 min. The ethanol was poured off the tube and the pellet was dried at 65 °C for 5 min. The DNA pellet was dissolved by adding 50 µl DW and heating at 65 °C for 5 min.

2.4. Optimizing PCR conditions

Two specific primers for PCR amplification of the 441 bp DNA fragment of alpha-Int1 gene (accession number: U35070.1) were designed using GenBank information and specific software. The pair primer sequences are as follows: (Forward c.a-Int1: 5′-AGGCAACTCCTAAAGCGTCA-3′. Reverse c.a-Int1: 5′-TGTTTTTCGAAGCGTCTTGC-3′.).

Generally, the designed pair of primers could amplify a 441 bp fragment of genomic DNA related to alpha-Int1 gene of *C. albicans*.

PCR mixture was prepared by adding the following ingredients: DDW: 14 µl, 10× buffer: 2.5 µl, $MgCl_2$ (50 mM): 0.75 µl, dNTP Mix (10 mM): 0.5 µl, forward primer (10 µM): 0.5 µl, reverse primer (10 µM): 0.5 µl, *Taq* polymerase (5 u/µl): 0.3 µl, templet DNA: 5 µl, with final volume of 25 µl.

The thermal profile for proper amplification of the target fragment was optimized as follows: primary denaturation of DNA: 94 °C, 3 min; cycle denaturation: 94 °C, 30 s; annealing at 62 °C for 30 s; extension at 72 °C for 60 s and final extension at 72 °C for 10 min, the process was repeated in 35 cycles to amplify the fragment.

2.5. Specificity and detection limits of the assay

ISO regulations recommend primary validation of any diagnostic assays through analytical sensitivity and specificity determination. To determine the specificity of the PCR assay genomic DNA(s) extracted from the following fungi strains was applied to PCR amplification tubes and processed in the same manner as done for *C. albicans* specimens: *F. solani*, *F. oxysporum*, *C. neoformans*, *S. cerevisiae*, *A. flavus*, *A. parasiticus* and *A. niger*.

To determine analytical sensitivity, the lowest detection limits of the assay, 10-fold serial dilutions from standard strains of *C. albicans* were prepared in DW and assayed by the PCR method for the presence of the alpha-Int1 DNA fragment.

2.6. Visualization of PCR amplification products

For visualization of PCR amplification products agarose gel electrophoresis (1.5%) was done using 25 mg of low-melting agarose added to proper volume of TBE buffer and equipped with 5 µl CyberGreen to stain DNA strands resolved on the gel. The final electropherogram was visualized in UV Transilluminator and recorded as a digital file for further analysis.

2.7. Sequencing of PCR-products

Dideoxy chain Termination sequencing reaction was used for sequencing of PCR products. Chromas LITE v:2.1.1 software was used to read and edit the nucleotide sequences and Mega 6 software was used to align the sequences.

3. Results

3.1. Optimization, specificity and sensitivity of the PCR assay

All suspensions containing *C. albicans* resulted in obvious electrophoretic bands related to PCR-amplified DNA fragments of alpha-Int1 gene (Fig. 1), showing application of the optimized primer set and thermal conditions through the assay. On the other hand, PCR amplification of DNA samples extracted from fungi strains other than *C. albicans* did not result in any visual electrophoretic bands that show 100% specificity of the assay for detection of *C. albicans* (Fig. 2).

For sensitivity of the assay, serial 10-fold dilutions from a *C. albicans* standard suspension were prepared in Distilled Water (DW) and genomic DNA content of dilutions was extracted and amplified. Electrophoretic analysis of the PCR products showed the assay is capable of detecting as many as 100 cells of *C. albicans* in the specimen.

3.2. Results of exposed samples

DNA content from radiation exposed and unexposed suspensions of *C. albicans* was extracted at given times and amplified using PCR assay. As shown in the Fig. 3, PCR amplification of DNA samples from both unexposed and 10, 70, 210, 350, 490 h radiation exposed yeast samples resulted in clear visible

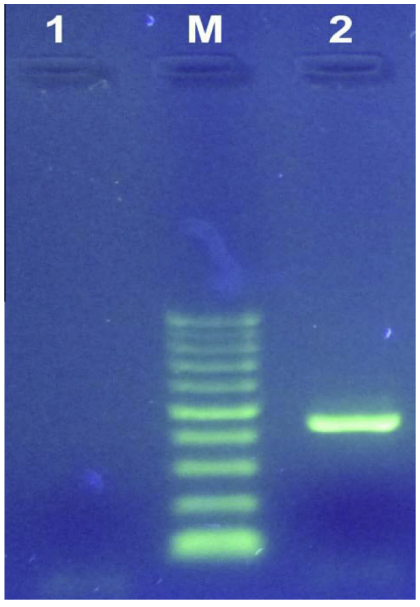

Figure 1 Agarose gel electrophoresis showing optimized conditions applied to PCR amplification of target DNA segment. M: 100 bp DNA ladder (vivantis), 1: PCR-amplification of DNA samples from negative control strains (fungi other than *Candida albicans*, see text) showing no band. 3: A clear band composed of DNA strands of 441 bp produced by PCR-amplification of DNA samples from positive control (*Candida albicans* only). The experiment was repeatedly done using a large number of negative and positive samples and Fig. 1 is a representative one.

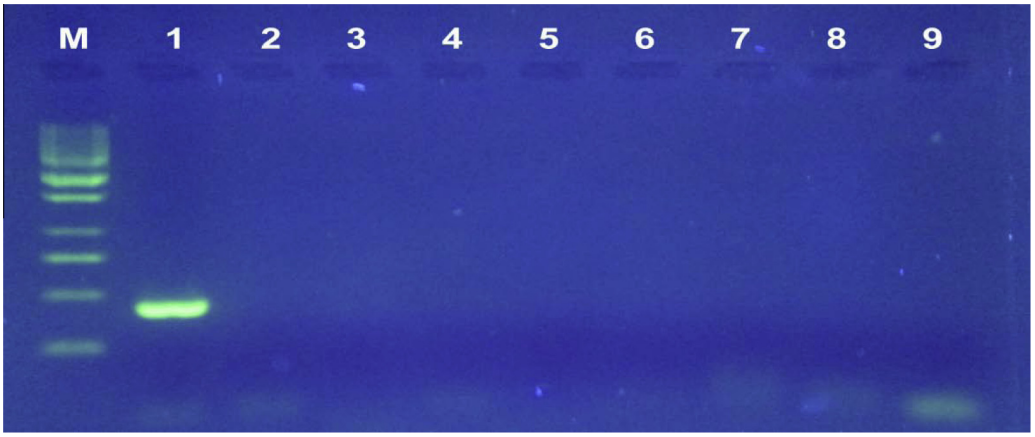

Figure 2 Agarose gel electrophoresis showing specificity of the PCR assay. PCR amplification of samples containing *Candida albicans* resulted in a clear band around 441 bp. PCR amplification of DNA samples from other fungi (lanes 2–8) did not result in any visible band. M: 1 kb DNA ladder (fermentas), 1: positive control (*Candida albicans* ATCC: 10231), 2: *Fusarium solani*, 3: *Fusarium oxysporum*, 4: *Cryptococcus neoformans*, 5: *Saccharomyces cerevisiae*, 6: *Aspergillus flavus*, 7: *Aspergillus parasiticus* and *8: Aspergillus niger*. The lane 9 belongs to the negative control.

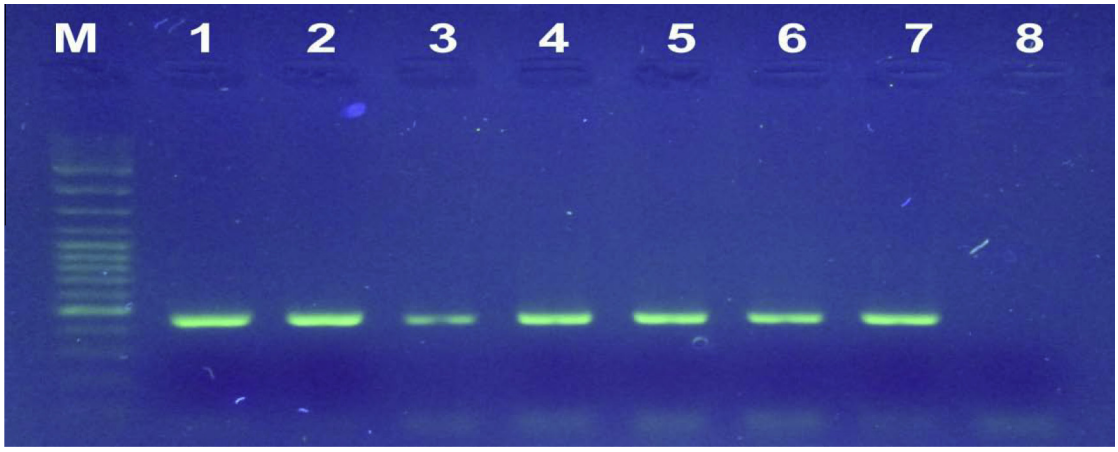

Figure 3 Electrophoresis of PCR amplification products of radiation exposed and unexposed yeast samples. All samples have resulted in clear bands representative of 441 bp segment of DNA related to alpha-Int1 gene. M: 1 kb DNA ladder (fermentas), lane 1: alpha-Int1 containing plasmid, lane 2: unexposed *Candida albicans*, lanes 3–7: 10, 70, 210, 350, 490 h radiation exposed yeasts, lane 8: negative control sample (DW lacking any DNA).

bands around 441 bp in the electropherogram. This means that 10–490 h exposure of *C. albicans* to 900 MHz radiation did not have any effect on alpha-Int1 gene sequence amplification through routine PCR assay.

3.3. Aligning sequenced PCR products

For aligning purposes, all PCR products from radiation exposed and unexposed samples were sequenced and read using Mega 6 software that has embedded tools like ClustalW or Muscle to align the sequence files. In general, 361 nucleotides (loci 659–1019, GenBank: U35070.1) of all sequenced DNA fragments obtained from exposed samples were aligned and compared with sequences obtained from unexposed samples. The results showed, Fig. 4, there are no nucleotide differences among exposed and unexposed samples regarding alpha-Int1 gene sequence. This means that long-term exposure (10–490 h) of *C. albicans* to 900 MHz mobile phone radiation did not have any mutagenesis effect (deletion, substitution or addition) on the nucleotide sequence of alpha-Int1 gene.

4. Discussion

Since May 2011, because of the new findings about close associations between long term mobile phone use and development of glioma, a brain tumor, the International Agency for Research on Cancer (IARC), has rated radiations from mobile phones in grade 2B, (available online at: <http://www.iarc.fr/en/media-centre/pr/2011/pdfs/pr208_E.pdf>. That means mobile phone radiation is a possible human carcinogen and mobile phone use could be associated with some risk of carcinogenicity (Deltour et al., 2012; Hardell et al., 2011; Hartikka et al., 2009; Little et al., 2012; Morgan, 2006).

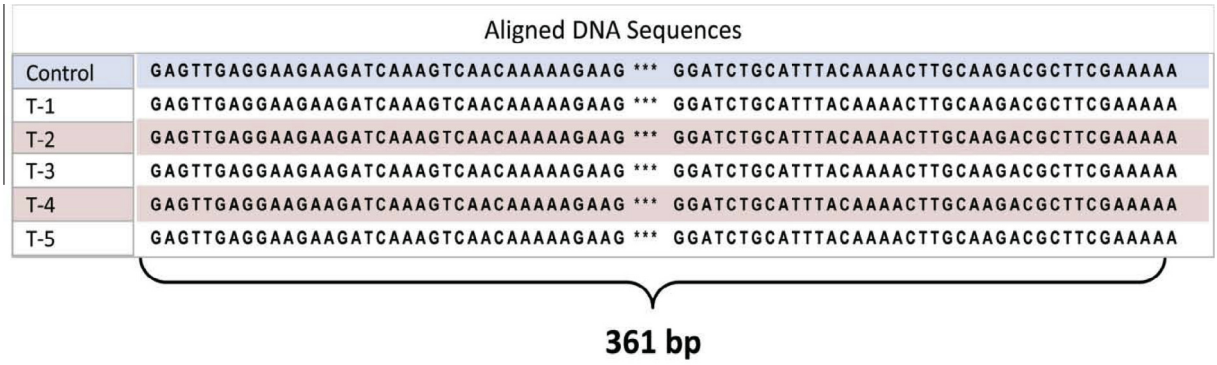

Figure 4 Aligning nucleotide sequences of alpha-Int1 gene produced through PCR amplification of exposed and unexposed samples. Nucleotide sequence presented in the first row (Control) is representative of results obtained from radiation unexposed samples. The remaining rows (5 rows presented here) belong to 900 MHz radiation exposed samples with the following exposure times: T-1: 10 h, T-2: 70 h, T-3: 210 h, T-4: 350 h, T-5: 490 h. Due to the large size of aligned sequence (361 nucleotides), only initial and end regions of the sequences were presented here. In general, long term exposure to radiation has had no mutagenic effect on alpha-Int1 gene sequence of *Candida albicans*.

Mobile phone safety standards are usually set by regulatory bodies considering the thermal effects of radiations from base stations and mobile handsets. Therefore, to minimize the thermal effects new generation mobile handsets usually operate at below 1 watt of energy. In addition to thermal effects, mobile phone radiation through different mechanisms may influence numerous biological systems including, disruption of cell membranes, altered gene expression, increased liability of cellular DNA to breakage by genotoxic agents (Belyaev et al., 2005), chromosomal abnormalities, micronucleus formation in the nucleus (Simko et al., 1998), altered chromatin structure (Markova et al., 2005), increased DNA fragmentation (De Iuliis et al., 2009), interruption of cell DNA repair system and increased incidence of tumors, induction of gene mutations (Pakhomov et al., 1998), dysfunction of essential enzymes (Ravera et al., 2011) and disturbed transmission of nerve impulses or disruption of transmembrane signaling of cells. Mobile phone radiations may also influence and increase the permeability of the blood–brain barrier (de Gannes et al., 2009; Eberhardt et al., 2008; Franke et al., 2005; Fritze et al., 1997; Leszczynski et al., 2002). Moreover, inactivation of peroxidase and alkaline phosphatase enzymes after the impact of radiations has been reported (Novikov et al., 1999). The previous study by Sadraei et al. (2004) evaluated using TEM the effects of 27.12 MHz electromagnetic radiation, 15 min twice daily for 7 days, on the bone ultrastructure of rat embryos. The results showed cytoplasmic vacuolization and shrinkage, degeneration of some organelles, nuclear condensation in the osteoblasts and decreased bony trabeculae in the extracellular matrix. In another survey by Ataee-Kachoi et al. (2004) have reported dual influences of shortwave and microwave radiations on toxin production by *Clostridium difficile*. Radiation exposure of the toxigenic bacterium inhibited toxin production while the exposure of the non-toxigenic strain donated the bacterium toxigenic potency.

Conversely, other studies have almost emphasized on harmlessness of the radiation. For example, Maes et al. (2001) have concluded through their experiments that 900 MHz radiations have had no mutagenic or co-mutagenic effects on cells and they also accented the radiation impacts on apoptosis of human lymphocytes are negligible (Maes et al. 2001). Jadidi et al. (2007) have studied the efficacy of Low-power density 950 MHz radiation on long-term potentiation (LTP) of the rat dentate gyrus. LTP is a long-lasting increase in synaptic efficacy resulting from the high frequency stimulation of afferent fibers. LTP is an attractive hypothesis to explain the cellular mechanisms of relational learning. The results showed that exposure to continuous GSM waves of 950 MHz EMF did not affect the LTP parameters. Other studies have even emphasized on beneficial impacts of radiations. Through another survey, Mansourian et al. (2012) examined the efficacy of mobile phone radiated electric field on survival of intact and cancerous cells under treatment of chemotherapy agents. The authors concluded that the 217 Hz electric field radiating from an operating mobile phone could significantly induce apoptosis in chemotherapy agent treated malignant cells while the field did not have any effects on healthy cells treated with the same agent. Therefore, the electric field could noticeably potentiate killing effect of chemotherapy agents. The authors did not present any explanation about the involving mechanisms (Mansourian et al., 2012).

The present study, for the first time, aimed at evaluating the mutagenic effect of microwave radiation on genome variation of *C. albicans*, a standard strain with ATCC number of 10231. The yeast was considered for the study because of its high prevalence in human population. In fact, the yeast is a habitual resident of the human skin and mucus membranes and the presence is not apparently associated with any symptoms. But in immunocompromised patients, including diabetics; organ transplant recipients and HIV positive persons, the yeast may cause several chronic or acute opportunistic infections including thrush, vaginitis, cutaneous and nail lesions, endocarditis, meningitis, encephalitis, arthritis and pyelonephritis.

Today, genus of candida accounted for 78.3% of the total nosocomial blood infections worldwide. *C. albicans*, is the most frequent species isolated from the infections, 59.8% out of 78.3% (Beck-Sague and Jarvis, 1993; Enfert and Hube, 2007; Ryan and Ray, 2004). *C. albicans* is also considered as the second agent, after *A. niger,* that can cause serious ear problems (Jadhav et al., 2003), especially among

immunocompromised patients (Viswanatha et al., 2012). The higher pathogenicity of *C. albicans* in humans, compared to other species of the genus of candida, is exactly related to the numerous virulence factors in the species.

The mucosal epithelial cell adherent capacity of *C. albicans* is considered as a virulence factor for the pathogen, and it has been shown that, the higher adherent potency of the yeast to the oral or gut mucus membranes is associated with the higher pathogenicity of the yeast (Gale et al., 1996). Several surface molecules from *C. albicans* are involved in the adhesion of the yeast to different target surfaces, and the participating molecules are varied according to the hydrophobic or hydrophilic nature of the target surfaces (Imbert et al., 2003). Some of these interactions may also require participation of additional ligand-receptor systems.

Alpha-Int1 is a frequently expressed molecule on the surface membrane of *C. albicans* and showed high structural similarity to the alpha subunit of the human leukocyte integrins. Alpha-Int1 molecule from yeast could interact with fibronectin and vitronectin molecules located on the epithelial and endothelial cells, or on the extracellular matrix (ECM). Therefore, the molecule plays an important role in the candida adherence to epithelial or endothelial cells. The alpha-Int1 gene expression is significantly increased through incubation of the yeast at 37 °C or in the presence of 10 mM glucose concentration (Santoni et al., 1994). Alpha-Int1, a transmembrane protein, is a polypeptide with molecular weight of 188-kDa composed of three extracellular, membrane-spanning and cytoplasmic regions and a tyrosine residue is located in the cytoplasmic end of the molecule. Transgene expression of the molecule in *Saccharomyces cerevisiae* gives the yeast the ability of germ tube production. Since germ tube production is an important virulence factor of *C. albicans*, it seems, the gene plays an important role in the yeast pathogenicity. Another study by Gale et al. (1998) shows the influence of alpha-Int1 gene expression level on *candida adherence* to tissue surfaces, filamentous growth and pathogenicity of the yeast. Lim and Lee (2000) revealed that the gene is the rapid detection of *C. albicans* in specimens through a PCR-based method. In the current study, the gene is used as a possible target sequence for microwave radiation and through the experiments we examined the hypothesis that, whether 900 MHz radiation could produce DNA breaks or any variation in the alpha-Int1 gene sequence.

As we reported in the result section, there were no obvious changes in the alpha-Int1 gene by this method. In other words, all DNA samples extracted from 10 to 490 h 900 MHz radiation exposed *C. albicans* suspensions, showed positive results, that means, the alpha-Int1 gene sequence remained unchanged after long term exposure of candida to radiation and produced reproducible results through the PCR amplification technique.

The result of the present study is similar to previous studies by other researchers, De Iuliis et al. (2009) and also Johansson et al. (2008). The researchers have examined different absorption doses and exposure times, and had an emphasis on the major influence of absorption dose and time on the outcome. In collection, they concluded that, lower absorption rate and lower exposure time are not associated with any tangible results (De Iuliis et al., 2009; Johansson et al., 2008). Other scientists have noticed the effect of different types of living systems that were exposed to radiation, to explain the controversies of the findings obtained from different studies. Chang et al. (2005) have reported a different outcome of radiation impact on different bacteria, as radiation targets.

In addition to the detection of any changes in the gene sequence that may influence its PCR amplification, whole alpha-Int1 gene was also sequenced, and again, there is no obvious variation in alpha-Int1 gene sequences obtained through sequencing of PCR amplification products of the different radiation durations of exposed candida samples.

Further explanation for the finding is the cell itself. *C. albicans* are the most resistant cells to radiation, even against X- or gamma radiations. This is due to the presence of potent repair systems and compensation mechanisms that enable the candida cells for rapid repair and regeneration of possibly broken DNA sequences and saving the intactness of the affected cells (Ciudad et al., 2004). Another factor that may influence and further reduce adverse effects of radiation on candida cells is the presence of a powerful anti-oxidant system in the cells. In fact, microwave radiation does not possess enough energy to directly cause DNA breaks in the nucleus, instead, the radiation triggers a strong oxidative stress inside the cells that finally leads to the release of free toxic radicals and DNA damage (Dasdag et al., 2009; De Iuliis et al., 2009; Kesari et al., 2011; Lu et al., 2012). Johansson et al. (2008), in his experiments, concluded that low intensity and low duration mobile phone radiation exposures are rapidly repaired by living cells and the anti-oxidant system of cells is a major compensatory mechanism against radiation triggered oxidative stress. Probably, other unknown factors also play significant roles in the resistance of cells to radiation induced damages. Nylund and Leszczynski (2006) have reported a noticeable influence of genome formulation and gene expression profile on radiation induced damages to different cells.

5. Conclusion

Results obtained from current study are emphasized on ineffectiveness of 900 MHz mobile phone radiation on alpha-Int1 gene amplification or sequence variation. The PCR method followed by product sequencing is an accurate method to analyse any sequence variation of genes, but the method is very expensive, time consuming and laborious. We examined a few hundred samples, but for accurate detection of a small number of mutated cells among large numbers of intact cells, the application of other inexpensive screening methods is necessary. Our failure in revealing radiation induced mutations, at least in our opinion, is partly due to the inability of PCR method to screen a large number of radiation exposed cells. In conclusion, other complementary studies, like real-time PCR method for the study of gene copy numbers and the level of expression, could exactly elucidate the true ineffectiveness of the radiation on gene variation.

Acknowledgments

This work was financially supported by Science and Research Branch of Islamic Azad University of Tehran. The study did not reach the ultimate without heartfelt adjutancies and technical guidance of our colleagues. We are grateful to Rasoul Soleimani for his assistance in preparing figures and editing the manuscript. Thanks are given for excellent technical assistance offered by Mahmoudi Mohammad-Amin, Mobaraki

Maryam, Faraji Abolfazl. Thanks are given to Dr. Akbari the top manager of genetic diagnosis lab for sequencing PCR products.

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