

**Original Paper**

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# Cell Death and Induced p53 Expression in Oral Cancer, HeLa, and Bone Marrow Mesenchyme Cells under the Exposure to Noncontact Electric Fields

Sahudi Abdul Mujib<sup>a</sup> Firman Alamsyah<sup>b</sup> Warsito Purwo Taruno<sup>b</sup>

<sup>a</sup>Medical School of Airlangga University and <sup>b</sup>Center for Medical Physics and Cancer Research of CTECH Laboratories Edwar Technology, Tangerang, Indonesia

## Keywords

Cell death · p53 expression · Cancer · Electro-Capacitive Cancer Therapy

## Abstract

**Background:** p53 acts as a transcription factor to regulate the expression of genes that modulate various cellular activities. The proliferation of cancer cells has been inhibited under the exposure to low-intensity (18 peak-to-peak voltage) and intermediate-frequency (100 KHz) electric fields generated between 2 capacitive electrodes. Therefore, the aims of this study were to observe the molecular mechanism of cell death caused by noncontact electric field exposure and to determine whether p53 protein can serve as a biomarker for this exposure or not. **Methods:** Oral squamous cell carcinoma, HeLa, and bone marrow mesenchyme cells were exposed to noncontact electric fields of Electro-Capacitive Cancer Therapy (ECCT) for 24 h. To observe the mechanism of cell death caused by ECCT, immunocytochemistry of p53 was performed, and the p53 expression was evaluated using immunoreactive score (IRS) calculation. **Results:** Electric field exposure by ECCT increased the percentage of dead cells in oral cancer cells (18.39%), HeLa cells (6.60%), and bone marrow mesenchyme cells (34.05%) with statistical significance using the independent *t* test compared to each control group. The IRS of p53 in oral cancer, HeLa, and bone marrow mesenchyme cultures were 10.50, 11.25, and 4.94, respectively. **Conclusion:** The high IRS shown in the treated oral cancer and HeLa culture cells may suggest that p53 expression in these culture cells is associated with the cell death mechanism induced by the exposure to noncontact electric fields, and the increased cell death in these culture cells may correlate with the IRS.

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## Introduction

The p53 protein is a tumor suppressor whose mutations are common features in various human tumors [1–3]. p53 is known to play a key role in all types of human cancers, and it is mutated in more than half of all human cancer cases worldwide [1, 4, 5]. This protein assesses deoxyribonucleic acid (DNA) damage and acts as a transcription factor regulating genes, which control cell growth, DNA repair, and apoptosis [6, 7], as well as senescence [8, 9]. Mutations of p53 gene cause the inactivation of the p53 protein so that cells cannot be arrested at the checkpoints of the cell cycle (G1 phase) or experience apoptosis, which, thus, leads to tumorigenesis resulting in DNA-damaged cells with a malignant phenotype [10, 11].

The proliferation of cancer cells has been inhibited, *in vitro* and *in vivo*, under the exposure to low-intensity (1–3 V/cm) and intermediate-frequency (100–200 KHz) electric fields generated between pairs of insulated electrodes contacting cells as well as tissues of animal models [12–15]. Cancer cells during mitosis contain highly polar, spatially oriented microtubules [16–19] and, thus, could be disoriented by the forces of electric fields [20, 21]. In treated cells under electric field exposure, mitosis began normally but, at the end, was extended to longer periods of time before cytokinesis [12, 22]. Furthermore, exposed cancer cells were destroyed as the formation of the cleavage furrow approached cytokinesis [12].

The normal mesenchyme cells have also been shown to respond to electric field exposure [23, 24]. Human mesenchyme cells which are grown in the stromal compartment of bone marrow are highly proliferative [25]. They have the ability to differentiate to become bone, cartilage, fat, and skeletal tissue and play prominent roles in fracture healing [23–27]. Electric fields are known to change the membrane morphology of mesenchyme cells [23, 28–30] and induce tissue responses, such as osteoblastic and osteogenic differentiation [26, 30, 31]. One possible cause for the membrane morphological change is the stress induced by the electric fields [32–38]. Therefore, we were interested in observing the effect of noncontact electric fields on mesenchyme cells as highly proliferative normal cells.

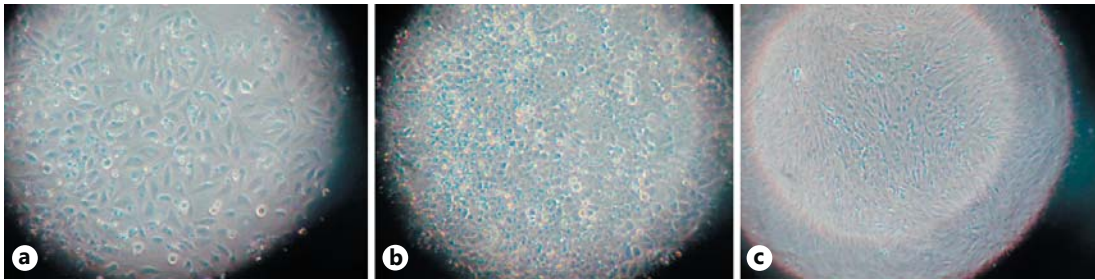
Previously, we have studied oral cancer in a clinical study, and we found that oral cancer cases in Indonesia are difficult to be cured [39]. Like oral cancer, cervix cancer is also difficult to be cured, and both types of cancer are among the top 10 cancers causing the highest mortality rate of cancer cases in Indonesia [40]. Therefore, we are searching for a new modality of cancer treatment for these 2 cancers. Thus, we were interested in observing the effect of noncontact electric fields on the proliferation of oral and cervix cancer cells.

We developed a noncontact electric field-based cancer therapy named Electro-Capacitive Cancer Therapy (ECCT) to inhibit the proliferation of breast cancer cells *in vitro* and *in vivo* using low-intensity (18 peak-to-peak voltage) and intermediate-frequency (100 KHz) electric fields generated between pairs of capacitive electrodes [41]. Although ECCT has successfully inhibited the proliferation of breast cancer cells, the molecular mechanisms of cancer cell death under the exposure to electric fields have never been studied before. Therefore, the aims of this study were to observe the molecular mechanisms of cell death caused by ECCT and to determine whether p53 protein can serve as a biomarker for this exposure or not. This is the first study in the existing literature describing p53 expression on cancer and normal cells under the exposure to low-intensity and intermediate-frequency noncontact electric fields.

## Materials and Methods

### *Cell Cultures*

This study was conducted using oral squamous cell carcinoma, HeLa, and bone marrow mesenchyme cell cultures (Fig. 1) obtained from cell banks of the Institute of Tropical Disease



**Fig. 1.** Oral cancer (a), HeLa (b), and bone marrow mesenchyme (c) cell cultures used in this study.



**Fig. 2.** A microplate flanked by 2 capacitive electrodes.

of Airlangga University. The cell cultures were thawed from cryopreservation and grown in  $\alpha$ MEM medium plus 20% fetal bovine serum, 1% penicillin-streptomycin, and 1% Fungizone in a carbon dioxide (CO<sub>2</sub>) incubator (5% CO<sub>2</sub>) at 37°C. One microliter cell suspension was placed in 24-well microplates loaded with 999  $\mu$ L medium (total:  $5 \times 10^5$  cells/mL). A pair of capacitive electrodes positioned flanking on top and at the bottom of the microplate was connected to a square function oscillator (Fig. 2). A one-directional field was generated between the pair of capacitive electrodes which alternated every 0.5 ms.

Cell cultures were treated with noncontact electric fields by ECCT and incubated for 24 h with 8 replications each. A control group with 8 replications was also incubated at the same time. At the end of the treatments, the dead cells were stained with trypan blue and counted using a microscope counting chamber (hemocytometer). The dead cell number was counted using this formula:

$$\% \text{ dead cells} = \frac{\text{total dead cells (stained)}}{\text{total cells (stained and unstained)}} \times 100.$$

The data were analyzed using the independent *t* test ( $p < 0.05$ ) and ANOVA test ( $p < 0.05$ ) run on SPSS version 20 to assess statistical significance.

#### *Immunocytochemistry*

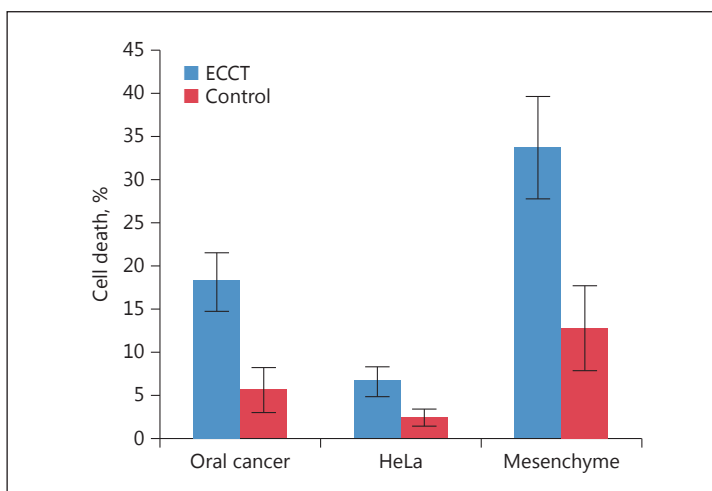
Immunostaining for p53 was performed on cells grown on coverslips beneath the wells of the microplates using UltraVision Detection System Anti-Polyvalent TP-015-HD (Thermo

**Table 1.** IRS scoring system [42]

Percentage of positive cells	X intensity staining	= IRS (0–12)
0 = no positive cells	0 = no color reaction	0–1 = negative
1 = <10% positive cells	1 = mild reaction	2–3 = mild
2 = 10–50% positive cells	2 = moderate reaction	4–8 = moderate
3 = 51–80% positive cells	3 = intense reaction	9–12 = strongly positive
4 = >80% positive cells		
IRS points	IRS classification	
0–1	0 = negative	
2–3	1 = positive, low expression	
4–8	2 = positive, intermediate expression	
9–12	3 = positive, high expression	

IRS, immunoreactive score.

**Fig. 3.** The percentage of cell death after treatment with ECCT compared to the control groups. ECCT, Electro-Capacitive Cancer Therapy.



Scientific) following the manufacturer’s protocol. The p53 expression shown by immunocytochemistry was evaluated using immunoreactive score (IRS) calculation by Remmele and Stegner (Table 1). The IRS was promoted for the first time for the immunohistochemical detection of estrogen receptors in mammary carcinoma [42]. The IRS evaluation was based on a modification of the evaluation of the visualized grade of color intensity (staining), and the fraction of cells in each intensity category was added [42].

## Results

The effect of ECCT on oral squamous cell carcinoma, HeLa, and bone marrow mesenchyme cell proliferation is illustrated in Figure 3. It demonstrates that the number of dead cells among the exposed cells (treatment group) is higher than that among the unexposed cells (control group). Electric field exposure by ECCT increased the percentage of dead oral cancer cells ( $18.25 \pm 3.36\%$ ), HeLa cells ( $6.66 \pm 1.77\%$ ), and bone marrow mesenchyme cells ( $33.75 \pm 5.80\%$ ) with statistical significance using the independent *t* test ( $p < 0.05$ ). Based on

**Table 2.** The number of cells in the treatment (T) and control (C) groups of oral cancer, HeLa, and bone marrow mesenchyme cells

Cell types	Cell condition	Cells of T group, <i>n</i>	Cells of C group, <i>n</i>
Oral cancer	Living cells	177,500.00±17,728.11	255,000.00±16,035.68
	Dead cells	40,000.00±10,000.00	15,625.00±7,288.69
HeLa	Living cells	778,125.00±81,017.53	942,500.00±28,535.69
	Dead cells	55,000.00±13,363.06	23,750.00±10,606.60
Mesenchyme	Living cells	115,000.00±12,535.66	132,500.00±17,113.07
	Dead cells	59,375.00±15,221.58	19,375.00±7,288.69

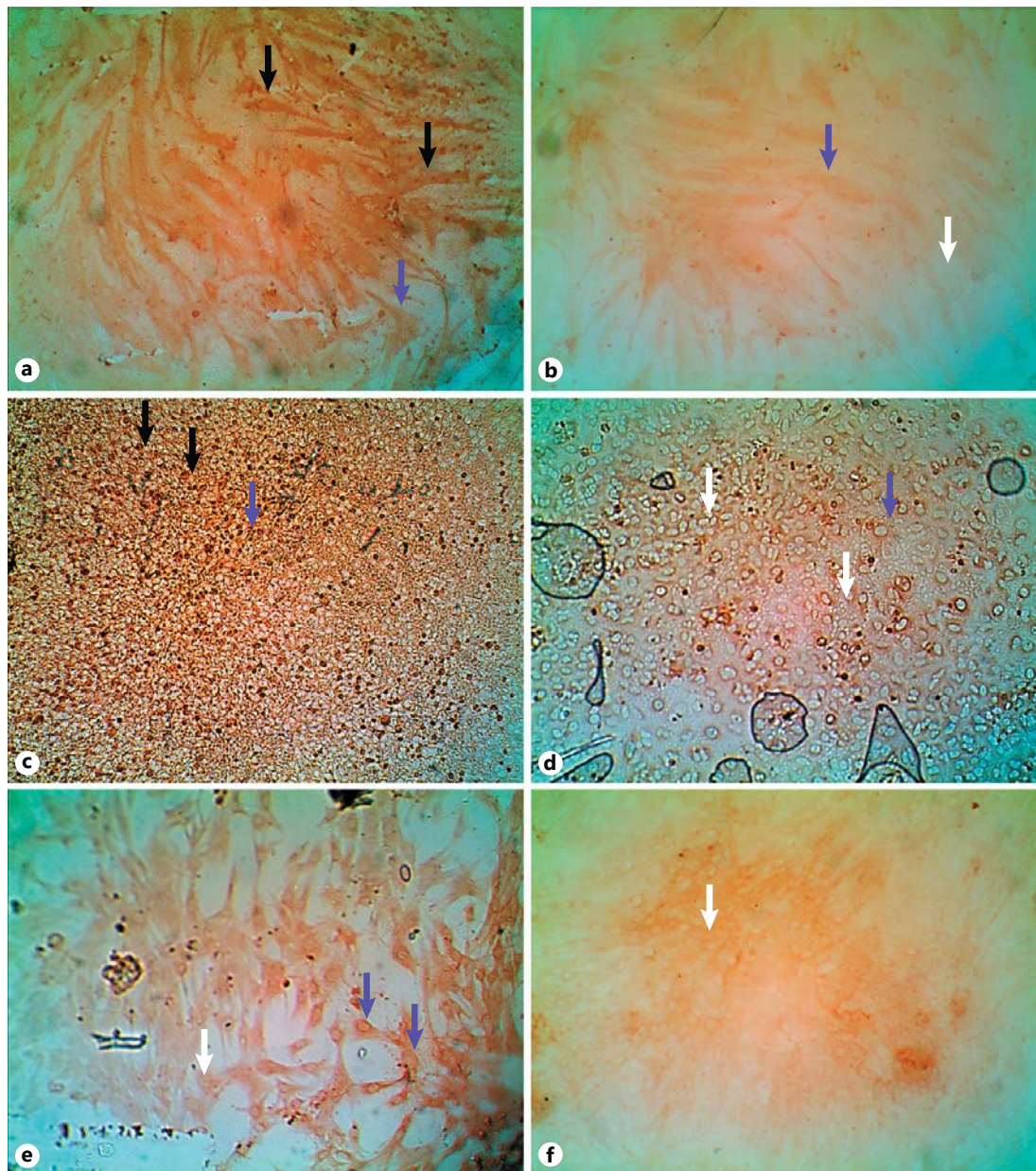
ANOVA test results ( $p < 0.05$ ), 24 h of exposure to an external electric field at 100 kHz caused the highest significant cell death in mesenchyme cells. The cell numbers of both the treatment and control groups are provided in Table 2.

To observe the mechanism of cell death caused by ECCT, immunocytochemistry staining of p53 was performed, and p53 expression on oral cancer, HeLa, and bone marrow mesenchyme culture cells was indicated by the brown color as seen in Figure 4. The IRS of p53 in oral cancer, HeLa, and bone marrow mesenchyme cultures was 10.50, 11.25, and 4.94, respectively.

## Discussion

Oral squamous cell carcinoma, HeLa, and bone marrow mesenchyme cell proliferation rates were decreased under the exposure to noncontact electric fields by ECCT. Two major processes take place at the cellular level during the exposure to electric fields: proliferation arrest and cell destruction. In treated cells under electric field exposure, mitosis that usually finishes within 1 h was extended to longer periods of time (on average within 2 h) before cytokinesis. This prolonged time of mitosis may eventually lead to cell death [12]. Moreover, exposed cancer cells were ruined as the formation of the cleavage furrow approached cytokinesis [13]. During this process, plasma membrane breakage took place and many small abnormal membrane blebs formed, corresponding to postmitotic apoptotic cell death [43, 44]. At the subcellular level, electric fields may interfere with the normal polymerization-depolymerization process of microtubules during mitosis [12, 45–47]. The inhibition of the proliferation of oral cancer and HeLa cells may suggest that treatment with low-intensity and intermediate-frequency noncontact electric fields by ECCT is quite effective and should be performed in an in vivo study on an animal model.

Like the proliferation of the cancer cells, the proliferation of the mesenchyme cells was also decreased under the exposure to noncontact electric fields by ECCT. The cell death of the mesenchyme cells did not only occur in the treatment group but also in the control group. This cell death can be explained by the aging process experienced by mesenchyme cells. Since the mesenchyme cells used in this study were derived from cell banks, they should show signs of in vitro aging [48, 49], including declines in cleavage capacity [49, 50] and replicative lifespan [49, 51, 52]. In addition, the maximal population doublings of mesenchyme cells in vitro are 30–40 times [49, 51, 53], and the average colony size decreases in aged mesenchyme cells [52, 54]. The exposure of mesenchyme cells to external electric fields also induced stress on the membrane of cells, thus increasing biomarker expression and stress response in mesenchyme cells. Heat shock protein 27 (hsp27) was upregulated under the exposure to



**Fig. 4.** p53 expression on oral, HeLa, and mesenchyme cell cultures. **a** Treatment oral cell cultures. **b** Control oral cell cultures. **c** Treatment HeLa cell cultures. **d** Control HeLa cell cultures. **e** Treatment mesenchyme cell cultures. **f** Control mesenchyme cell cultures. Black, blue, and white arrows point to cells with p53 high, intermediate, and low expression, respectively.

electric fields as a stress response of mesenchyme cells [26, 55, 56]. hsp27 impeded p53-mediated accumulation of p21, the main regulator of cellular senescence [57–60], and, therefore, suppressed cellular senescence by modulating the p53 pathway [61–64]. This report corresponds to the moderate IRS of mesenchyme cells (4.94). The molecular mechanism of mesenchyme cell death under the exposure to electric fields by ECCT will be further investigated. Moreover, the differences in the cell death rate between the 3 cell cultures caused by the different response of each cell to the electric fields need to be explained. It has

been suggested that in vitro treatment duration should vary between cell lines and correspond to their cell doubling time [65].

In the p53 expression study of cancer cells under the exposure to low-intensity and intermediate-frequency noncontact electric fields, we found 80–85% positively stained cancer cells for p53 protein on oral and HeLa culture cells. Consequently, we can assume that this overexpression of p53 is related to the ECCT exposure. We have mentioned earlier that external electric field exposure disturbs the proper formation of the mitotic spindle formed by the polymerization of microtubules. This disruption ultimately turns on the spindle assembly checkpoint, also known as the mitotic checkpoint, whose role it is to maintain genome stability by arresting cell division from metaphase to anaphase to prevent abnormal segregation of sister chromatids [66–69] or by stirring apoptosis in a way identical to that reported in studies with typical antimicrotubule agents [65, 70, 71]. Moreover, cell death can happen subsequently to cell division. For example, arrested cell division can be followed by senescence or apoptosis occurring in the next G1 phase [65, 72–74]. Mitotically arrested cells can finish mitosis, then entering G1 phase as aneuploid cells in a process called mitotic slippage [65, 73, 75, 76]. Cell death via apoptosis was one of the possible outcomes of mitotic slippage [65, 75, 76]. Thus, a postmitotic cell death which involves activation of the p53 pathway is possible [65, 76–78].

Aneuploidy derived from mitotic slippage caused genomic instability, since it showed an abnormal number of chromosomes [76, 78, 79], and subsequent cell death or senescence in a process known as mitotic catastrophe [65, 74, 80]. In addition, cell death induced by electric fields was caspase dependent, but the first triggers of the caspase-dependent apoptosis were not known [65]. Aneuploidy generates increased levels of energy metabolism as well as intracellular reactive oxygen species, which cause oxidative DNA damage and ataxia-telangiectasia mutated kinase (ATM) activation [77, 81, 82]. The ATM is a kinase that phosphorylates p53, which results in the activation of p53 [77, 83, 84]. In addition, p38 stress kinase also phosphorylates p53 in response to chromosome mis-segregation, which eventually mediates cell cycle arrest [76, 78, 85]. Moreover, in mouse embryonic fibroblasts, which are greatly aneuploid and grow poorly, p53 was found to be activated [77]. The activation of p53 resulted in the activation of p21 for delaying the cell cycle [11, 77, 78, 86] and caspase 3 for apoptosis [77, 87, 88]. Caspase 3 activation might elucidate the embryonic lethality of mutant mice and the high apoptosis rates in mouse embryonic fibroblasts derived from these mice [77, 87]. Therefore, the exposure to noncontact electric fields by ECCT may result in aneuploid cells that activate p53, as shown by the high IRS, and may eventually induce cell cycle arrest and caspase-dependent apoptosis. Since p53 alone is not sufficient to conclude any molecular pathway, the molecular pathway of cell cycle arrest and caspase-dependent apoptosis in aneuploid cells mediated by p53 under the exposure to ECCT electric fields needs to be further investigated.

## Conclusion

Finally, from this study we can conclude that p53 may serve as a biomarker for the exposure to noncontact electric fields by ECCT, and the high expression of p53 on cancer cells may be associated with the cell death mechanism induced by the exposure to ECCT.

## Disclosure Statement

The authors declare no conflicts of interest.

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