www.ijrps.pharmascope.org

ISSN: 0975-7538
Research Article

# Expression pattern of pro- and anti-apoptotic proteins in patients with squamous cell carcinoma of the uterine cervix

**Krishnan Baskaran*[1], Ramanathan Satish[1], Viswan Santhosh[1], Radhika Govindaraju[1], Shanmugam Manoharan[2], Subramanian Sethupathy S[1]**

[1]Division of Biochemistry, Rajah Muthiah Medical College, Annamalai University, Annamalainagar-608 002, Tamil Nadu, India
[2]Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar-608 002, Tamil Nadu, India

**ABSTRACT**

Apoptosis is a complex pathway, which plays a central role in the physiological processes. Aberrant regulation of apoptosis or defective expression of apoptotic- related proteins might result in tumourigenesis. The present study investigated the expression pattern of Bcl-2 and Bax by immunohistochemistry and the activities of caspase-3 and -9 by ELISA reader in patients with squamous cell carcinoma (SCC) of the uterine cervix. Bcl-2 expression was found in 65% of the tumour cells while Bax expression was found in 45% of the tumour cells. The activity of caspase-3 was significantly reduced ($p<0.001$) while the activity of caspase-9 was unaltered in patients with SCC ($p>0.05$) as compared to normal subjects. The results thus suggest defective expression of pro- and anti- apoptotic proteins in patients with SCC of the uterine cervix, which could play a role in the abnormal cell proliferation of the uterine cervix.

**Keywords:** Apoptosis; Bcl-2; Bax; Caspase; Cervical cancer.

## INTRODUCTION

Cancer of the uterine cervix is the most common cancer affecting women worldwide (WHO, 2006). The estimated global incidence is around 500,000 and nearly half of the women die of this cancer every year (Parkin et al., 2005). In the United States, approximately, 11,000 new cases and 4000 deaths due to this cancer reported in 2009 (Jemal et al., 2009). In India, it is the most common cancer among women and approximately 90,000 cases were newly reported in 2007 (NCRP, 2009).

Apoptosis, a highly organised complex pathway, is essential to eliminate unwanted cells in the development and homeostasis of multicellular organisms (Shehata, 2005). Aberrant regulation of apoptosis has been implicated in the pathogenesis of number of clinical disorders (Donepudi et al., 2002). Enhanced apoptosis could lead to ischaemic and neurodegenerative diseases while insufficient apoptosis could lead to autoimmune and malignant disorders (Donepudi et al., 2002). Apoptosis is induced when there is irreparable DNA damage or when cell division goes out of control. It occurs by the activation of a proteolytic cascade of apoptosis specific enzymes, the caspases (Thornberry et al., 1998). So far, 14 mammalian caspases have been identified and 12 of them are found in human (Oliver et al., 2005). Based on the mode of activation, the pro-apoptotic caspases are categorised as the upstream signaling group initiator or apical caspases (caspase-2, caspase-8, caspase-9 and caspase-10) and downstream effector caspases (caspase-3, caspase-6 and caspase-7) (Shi, 2002). All caspases are produced in cells as catalytically inactive zymogens and must undergo activation during apoptosis (Donepudi et al., 2002). The activation of an effector caspase such as caspase-3 or 6 is performed by an initiator caspase such as caspase-9. However, the initiator caspases are auto-activated depending on the apoptotic signals or stimuli (Shi, 2002). Once activated, the effector caspases exert various actions on the cells ultimately leading to cell death (Shehata, 2005). The actions include break down of structural components like actin and nuclear lamin, inactivation of DNA repair enzyme like poly (ADP-ribose) polymerase (PARP), and activation of caspase activated DNase (CAD) which degrades chromosome in to nucleosomal fragments during apoptosis (Shehata, 2005).

Bcl-2 family of proteins, the key regulator of apoptosis, consists of both anti-apoptotic (Bcl-2, Bcl-$X_L$, Bcl-W, Mcl-1, A1) and pro-apoptotic members (Bax, Bak, Bcl-$X_S$, Bad, Bid) (Danial, 2007). These pro- and anti-apoptotic proteins can homodimerise or heterodime-

* Corresponding Author
Email: jasibaskaran@rediffmail.com
Contact: +91-4144-238248
Received on: 29-09-2010
Revised on: 12-12-2010
Accepted on: 14-12-2010

rise and neutralise one another's functions (Korsmeyer, 1999). The most important pro-apoptotic protein is Bax and the most important anti-apoptotic protein is Bcl-2. The expression of both Bcl-2 and Bax has been described in cervical carcinomas with conflicting results (Tjalma et al., 2001; Chung et al., 2002; Ohno et al., 1998). Furthermore, there are only a few studies available on the activities of caspase-3 and -9 in cervical carcinomas (Chung et al., 2002; Aréchaga-Ocampo et al., 2008). Thus our aim was to investigate the expression pattern of Bcl-2, Bax, caspase-3 and -9 in patients with squamous cell carcinoma of the uterine cervix.

## MATERIALS AND METHOD

### Study design

Twenty subjects, clinically evaluated as having cervical cancer, ranging in age from 30- 65 years were selected for the present study. Tumour samples were obtained from patients admitted in Obstetrics and Gynaecology Division at Rajah Muthiah Medical College and Hospital, Annamalai University, Annamalainagar. The patients were staged according to FIGO (International Federation of Gynaecology and Obstetrics) staging and stage III was selected for the present study. All the tumour samples were squamous cell carcinomas confirmed by histopathological examination. Twenty age matched normal subjects were also investigated for the present study. They were undergoing hysterectomy for uterine prolapse. The informed consent was obtained from all the study subjects and the study was approved by the Institutional Human Ethical Committee (IHEC). Biopsy samples were collected by gynaecologist. The specimens were divided into two parts. One part was washed with normal saline and used for homogenisation. The other part was placed in neutral buffered formalin (10%) and embedded in paraffin within 24 hours for long time storage. Several 4 µm sections were cut from each tissue specimen for conventional light microscopic as well as immunohistochemical analyses.

### Preparation of tissue homogenate

Cytosolic extracts were prepared by homogenising cervical tissues in lysis buffer containing 50 mM 2-[4-(2-hydroxylethyl)-1-piperazinyl] ethane sulphonic acid(HEPES; pH 7.4), 5mM 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulphonate (CHAPS) and 5mM dithiothreitol (DTT). Homogenate was centrifuged in a microcentrifuge (10,000 x g) for 1 minute. The supernatent (cytosolic extract) was collected as an enzyme source. The protein content of the supernatant was estimated using Bradford reagent (Bradford, 1976).

### Assay of caspase-3 and -9

The activity of caspase-3 was assayed in tissue lysates using caspase-3 colourimetric assay kit (Biovision, USA) according to the manufacturer's instructions. The caspase-3 colourimetric assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage from the labeled substrate acetyl-Asp-Glu-Val-Asp-nitroanilide (Ac-DEVD-pNA) by caspase-3. The free p-nitroaniline is quantified using ELISA reader at 405 nm. Similarly, the activity of caspase-9 was assayed using caspase-9 colourimetric assay kit (Biovision, USA).

### Immunohistochemistry

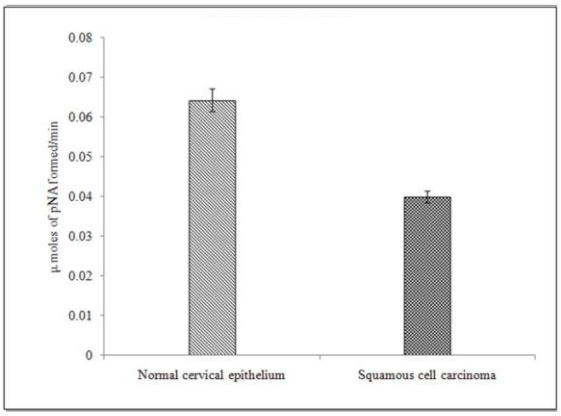
Paraffin tissue sections were fixed on pre-coated slides [(BioGenex OptiPlus$^{TM}$) BioGenex, San Ramon, CA, USA]. Paraffin was removed from the slides by heating at 60°C for 10 min and then washed three times in xylene for 10 min each. After gradual hydration through graded alcohols, the sections were rinsed in distilled water for five minutes. All slides were incubated for 30 min in 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity. The antigen retrieval was achieved by adding citrate buffer (pH 6.0) and keeping in pressure cooker for 10 minutes. The tissue sections were then incubated with universal proteinaceous blocking reagent power Block$^{TM}$ (BioGenex, San Ramon, CA, USA) for 15 min at room temperature to prevent non-specific binding. For localisation of the Bcl-2 and Bax proteins, the sections were incubated with a respective ready to use primary antibodies: anti-Bcl-2 oncoprotein – mouse monoclonal Ab in PBS; Anti-Bax protein – Rabbit polyclonal Ab in PBS (BioGenex, San Ramon, CA, USA ) for 60 min at room temperature. The sections were then incubated with their corresponding secondary antibodies, polymer HRP detection system (BioGenex, San Ramon, CA, USA) conjugated with horseradish peroxidase for 30 min at room temperature. The antigen-antibody complex was detected using 3, 3´-diaminobenzidine (Sigma, St Louis, MO, USA). The immunostained slides were counterstained with mayer's hematoxylin for 5 min and covered with a mounting medium.

### Assessment of immunostaining

All slides were evaluated for immunostaining without the knowledge of clinical data. A section was considered as positive when ≥ 10% of the tumour cells showed immunoreactivity. This was obtained by estimating the fraction of positive tumour cells in several random fields (400×) and averaging the results. In the present study, no attempt was made to grade the staining intensity. This was adopted from the earlier publication by Chung et al (2002).

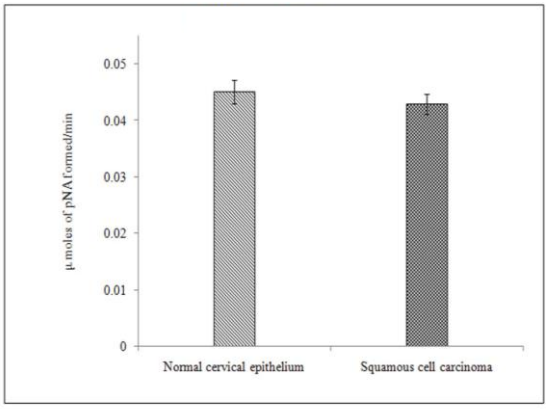
## RESULTS

The present study investigated the activities of caspases-3, and -9 using DEVD-specific colourimetric assay kit and immunohistochemical expression of Bcl-2 and Bax in 20 squamous cell carcinoma specimens and 20 normal cervical tissues. The activity of caspase-3 was significantly reduced in cervical cancer as compared to controls (Fig.1a). The activity of caspases-9 was not significantly altered in cervical cancer as compared to




Values are expressed as mean ± SD: (P < 0.001)

**Figure 1a: Caspase-3 activity**




Values are expressed as mean ± SD: (P > 0.05)

**Figure 1b: Caspase-9 activity**

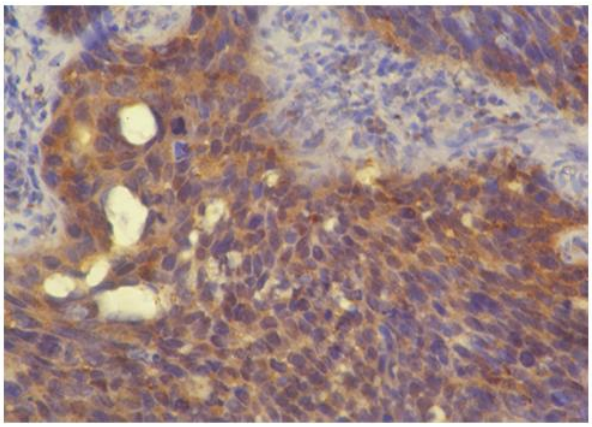


**Figure 2a: Cytoplasmic staining of bcl-2 (400x)**

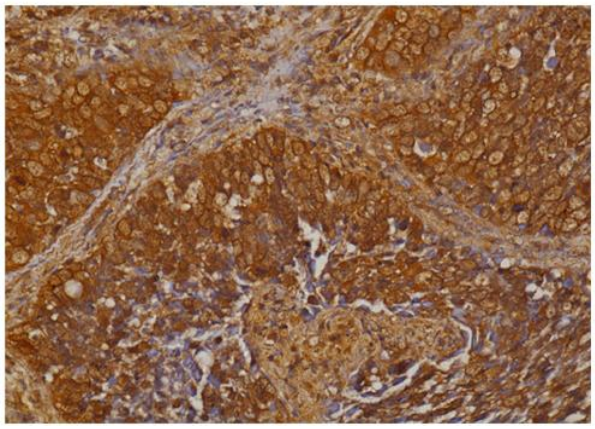


**Figure 2b: Cytoplasmic staining of bax (400x)**

controls (Fig.1b). Immunostaining for Bcl-2 was localised in the cytoplasm of the cells. The protein expression was heterogeneous and present in 13 (65%) squamous cell carcinoma (Fig.2a) and in eight (40%) normal cervix. Immunostaining for Bax was cytoplasmic. The protein expression was present in nine (45%) squamous cell carcinoma (Fig.2b). Only four (20%) of the normal cervix were immunopositive for 10% cut off value.

**Statistical analysis**

Data for the activities of caspase-3 and -9 are expressed as mean ± SD. Statistical analysis was carried out using a Student's *t* test. The results were considered statistically significant if the value was 0.05 or less.

**DISCUSSION**

The normal cellular growth is a complex process, which involves a fine balance between proliferation and apoptosis. The net accumulation of cells and thus cancer development can occur as a consequence of enhanced proliferation or diminished apoptosis. Almost all types of human cancers are found to be resistant to apoptosis (Hanahan et al., 2000) and many alterations in apoptotic machinery have been observed in human cancers (Aréchaga-Ocampo et al., 2008).

There have been limited studies on the activities of caspases-3 and -9 in cervical carcinomas. However, few studies used immunohistochemical technique on tissue caspases in cervical carcinomas (Chung et al., 2002; Aréchaga-Ocampo et al., 2008). We employed colourimetric technique to measure the activities of these caspases. Our study showed that the activity of caspases- 3 was significantly reduced ($p<0.001$) where as the activity of capase-9 was not significantly altered in patients with squamous cell carcinoma as compared to subjects with normal cervix.

Caspases are the final mediators of apoptosis and they bring the culmination of the programmed cell death. Activation of caspase cascade is necessary for the onset of apoptosis in mammalian cells (Wolf et al., 1999). Defective activation or expression of caspases has been implicated in tumourigenesis of various human cancers (Philchenkov et al., 2004). Aréchaga-Ocampo et al (2008) reported that the expression of caspases-3 and -9 was undetectable in adenocarcinoma and adenosquamous cell carcinomas. However, in squamous cell carcinoma of the uterine cervix, the expression of these enzymes was similar to those observed in normal cervix. Cheung et al (2002) reported reduced immunohistochemical expression of caspase-3 in squamous cell carcinoma of the uterine cervix as compared to high-grade squamous intraepithelial lesion (HSIL). Our study

demonstrated the reduced activity of caspase-3 and unaltered activity of caspase-9 in squamous cell carcinoma.

In the present study, the expression of Bcl-2 protein was up regulated in squamous cell carcinoma (65%) as compared to normal cervical epithelium (40%). Our results are in agreement with Chung et al (2002) who reported 65% expression of Bcl-2 for 10% cut-off value. Tjalma et al (1997) reported 63% Bcl-2 expression for 5% cut-off value. The up regulation of Bcl-2 is suggestive of defective apoptosis in squamous cell carcinoma. Studies have shown that Bcl-2 over expression could block apoptosis and prolong cell survival without altering cell proliferation (Reed, 1994). Nair et al (1999) demonstrated up regulation of Bcl-2 by immunohistochemical analysis and reduced apoptosis in squamous cell carcinoma of the uterine cervix by using *in situ* technique.

In immunohistochemical analysis, the expression of Bax was 45%. Our results are comparable to Kokawa et al (1999) who observed 44 % Bax expression in squamous cell carcinoma. Van de Putte et al (2005) reported 82% while Ohno et al (1998) observed 15% in cervical cancer. The relation between Bax and Bcl-2 proteins has been described as a cellular rheostat and ratios of the two proteins determine the state of apoptosis (Korsmeyer, 1999). When Bax is over expressed it homodimerises itself and promotes apoptosis, conversely when Bcl-2 is over expressed, it heterodimerises with Bax and suppresses apoptosis. (Korsmeyer, 1999). The present study observed reduced Bax to Bcl-2 ratio and this might have resulted in the tumourigenesis (Nair et al., 1999).

## CONCLUSION

Our results showed defective expression of pro-and anti- apoptotic proteins in patients with squamous cell carcinoma of the uterine cervix, which could play a possible role in the abnormal cell proliferation of the uterine cervix. Further studies involving other members in the Bcl-2 family would throw more light on the role of apoptotic machinery in the squamous cell carcinoma of the uterine cervix.

## REFERENCES

Aréchaga-Ocampo E, Pereira-Suárez AL, del Moral-Hernández O, et al. HPV+ cervical carcinomas and cell lines display altered expression of caspases. Gynaecol Oncol, vol. 108, 2008, pp. 10-18.

Bradford M. A rapid and sensitive method for the quantification of microgram quantity of protein utilising the principle of protein dye binding. Anal Biochem, vol. 72,1976, pp. 248-254.

Cheung TH, Chung TK, Lo KW, et al. Aoptosis-related proteins in cervical intraepithelial neoplasia and squamous cell carcinoma of the cervix. Gynaecol Oncol, vol.86, 2002, pp.14-18.

Chung. TK, Cheung. TH, Lo.WK, et al. Expression of apoptotic regulators and their significance in cervical cancer. Cancer Lett, vol. 180, 2002, pp. 63-68.

Danial NN. Bcl-2 family proteins: critical checkpoints of apoptotic cell death. Clin Cancer Res, vol.13, no.24, 2007, pp. 7254-7263.

Donepudi M and Grütter MG. Structure and zymogen activation of caspases. Biophys Chem, vol.101-102, 2002, pp. 145-153.

Hanahan D. and Weinberg RA. The hallmarks of cancer. Cell, vol. 100, 2000, pp. 57- 70.

Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2009. CA Cancer J Clin, vol. 59, no. 4, 2009, pp. 225-249.

Kokawa K, Shikone T, Otani T and Nakano R. Apoptosis and the expression of Bax and Bcl-2 in squamous cell carcinoma and adenocarcinoma of the uterine cervix. Cancer, vol. 85, no. 8, 1999, pp. 1799-1809.

Korsmeyer SJ. Bcl-2 gene family and the regulation of programmed cell death. Cancer Res, vol.59, 1999, pp. 1693s-1700s.

Nair P, Nair MK, Jayaprakash PG and Pillai MR. Decreased programmed cell death in the uterine cervix associated with high risk human papillomavirus infection. Pathol Oncol Res, vol. 5, no. 2, 1999, pp. 95-103.

National Cancer Registry Programme (NCRP, ICMR). Time trends increase incidence rates: 1982- 2005. Bangalore: NCRP, 2009.

Ohno T, Nakano T, Niibe Y, Tsujii H and Oka K. Bax protein expression correlates with radiation-induced apoptosis in radiation therapy for carcinoma. Cancer, vol.83, no. 1, 1998, pp.103-110

Oliver L and Vallette FM. The role of caspases in cell death and differentiation. Drug Resist Updat, vol. 8, 2005, pp. 163-170.

Parkin DM, Bray F, Ferlay J and Pisani P. Global cancer statistics, 2002. CA Cancer J Clin, vol.55, 2005, pp. 74-108.

Philchenkov A, Zavelevich M, Kroczak TJ and Los M. Caspases and cancer: mechanisms of inactivation and new treatment modalities. Exp Oncol, vol. 26, no. 2, 2004; pp. 82-97.

Reed JC. Bcl-2 and the regulation of programmed cell death. J Cell Bio, vol.124, 1994, pp.1-6

Shehata MF. Rel/Nuclear factor–kappa B apoptosis pathways in human cervical cancer cells. Cancer cell Int, vol.5, no.10, 2005, pp.1-13.

Shi Y. Mechanisms of caspases activation and inhibition during apoptosis. Mol cell, vol. 9, 2002, pp. 459-470.

Thornberry NA and Lazebnik Y. Caspases: enemies within. Science, vol 281, 1998 pp.1312-16.

Tjalma W, Weyler J, Bogers J, et al. The importance of biological factors (Bcl-2, Bax, p53 PCNA, MI, HPV and angiogenesis), in invasive cervical cancer. Eur J Obstet Gynaecol Reprod Biol, vol. 97, 2001, pp. 223-230.

Tjalma W, Weyler.J, Goovaerts G, et al. Prognostic value of Bcl-2 expression in patients with operable carcinoma of the uterine cervix. J Clin Pathol, vol. 50, no.1, 1997, pp. 33-36.

Van de Putte G, Holm R, Lie AK, et al. Markers of apoptosis in stage IB squamous cervical carcinoma. J Clin Pathol, vol. 58, 2005, pp. 590- 594.

Wolf BB and Green DR. Suicidal tendencies: apoptotic cell death by caspases family proteinases. J Biol Chem, vol. 274, no. 29, 1999, pp. 20049-20052.

World Health Organisation. Comprehensive Cervical Cancer Control: a guide to essential practice. Geneva, Switzerland, WHO; 2006.