

# The correlation of protein peroxidation with morphological changes in experimental oestradiol-induced carcinogenesis

Jarosław Kobiela<sup>1, 2</sup>, Jolanta Kubasik-Juraniec<sup>4</sup>, Tomasz Stefaniak<sup>2</sup>, Jacek Krajewski<sup>2</sup>, Guido Bellezza<sup>3</sup>, Emilio Bucciarelli<sup>3</sup>, Paulina Kryger<sup>1</sup>, Andrzej Lachinski<sup>2</sup>, Zbigniew Gruca<sup>2</sup>, Jan H. Spodnik<sup>5</sup>, Michał Woźniak<sup>1</sup>

<sup>1</sup>Department of Medical Chemistry, Medical University, Gdańsk, Poland

<sup>2</sup>Department of General, Gastroenterological and Endocrinological Surgery, Medical University, Gdańsk, Poland

<sup>3</sup>Laboratory of Electron Microscopy, Medical University, Gdańsk, Poland

<sup>4</sup>Pathological Anatomy and Histology, Division of Cancer Research, Perugia University, Policlinico Montelucente, Perugia, Italy

<sup>5</sup>Department of Anatomy and Neurobiology, Medical University, Gdańsk, Poland

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*Oestradiol-induced male Syrian hamster carcinogenesis is a well-known experimental model of human cancer of the breast, ovary and uterus. The pathomechanism postulated in this model is 4-hydroxylation of oestradiol and further free radical formation. The same process is suspected in human breast cancer. Dynamic changes in protein peroxidation were reported during the tumour induction. In this paper we try to correlate the protein peroxidation markers with the histopathological progression of the changes. The biochemical and histopathological evaluations were performed after 1, 3, 6 and 9 months of the hormone exposition. Significant protein peroxidation was observed as soon as after 1 month and increased further until the 6th month. After 9 months however, it was not significantly different from the control. The discrete histopathological changes after 1 month, progressed into tubular and interstitial hyperplasias after 3 and 6 months. After 9 months several dysplastic areas, sometimes with features of carcinoma in situ, were observed. The severe 9-month histopathological changes did not correlate with the protein peroxidation.*

**key words:** oxidative stress, protein carbonyl groups, renal carcinogenesis, estrogen-induced carcinogenesis, morphology

## INTRODUCTION

Experimental oestradiol-dependent kidney cancer has recently become one of the most exploited models of experimental carcinogenesis. This is due to its high applicability in research into the early stages and pathomechanism of human hormone-dependent cancers, predominantly breast cancer. As the incidence of breast cancer is rapidly increasing in developed western countries, the importance of pre-

vention and treatment has become one of the chief interests of carcinogenesis-orientated scientists.

Oestrogen-dependent cancer of the kidney of male hamsters was first described by Kirkman in 1959 [6]. Since then many researchers have studied its biochemical and molecular pathogenesis. The theory postulated by Liehr et al. [8, 11, 18] indicates the activity of semiquinone and quinone-free radicals originated in redox cycling of 4-OH-oestradiol deriv-

atives. The formation of 4-OH-oestradiol from oestradiol is catalysed by oestradiol 4-hydroxylase [10]. The activity of this enzyme is increased in oestradiol carcinogenesis target organs such as the human breast, the human uterus, mice uterus and the kidney of the male hamster. The activity of oestradiol, described as epigenotoxic, includes direct lesions of DNA and proteins by oestradiol semiquinone and oxygen-free radicals derived from semiquinone-quinone transmission, and further mitotic stimulation of neoplastic cell lines.

Although the pathogenesis of the cancer is becoming increasingly clear, its histopathology still poses some open questions. Cortes-Vizcaino and Llobart-Bosch [3] and Cortes-Vizcaino et al. [4] have determined the cancer as originating from blastemal cells and further differentiating into epithelial or interstitial pathways Bhat et al. [2] and Hacker et al. [5] observed the presence of vimentin and desmin in the neoplastic foci, which suggested that the tumour originates from the perivascular smooth muscles [2, 5]. Oberley et al. [14–16] described the early changes as originating from interstitial cells and defined them as interstitial hyperplasia or tubular dysplasia. He found the better expression of vimentin and desmin (not keratin) in preneoplastic lesions. Interestingly the advanced stage oestrogen-induced renal tumour revealed strongly positive vimentin, desmin and keratin staining. This finding would support the idea of Cortes-Vizcaino and Llobart-Bosch [3].

As was mentioned above, the peroxidative activity of oestradiol and its derivatives seems to play a crucial role in the development and maintenance of oestrogen-dependent male Syrian hamster kidney cancer. Despite this, none of the authors have presented a clear correlation between the intensity of oxidative stress (or oxidative damage) and the extent of the preneoplastic or neoplastic kidney lesions. The dynamics of the protein peroxidative damage has recently been presented in the model by Stefaniak et al. [19]. The current paper presents the results of a study comparing the intensity of oxidative stress with pathomorphological changes observed at consecutive stages of carcinogenesis.

## MATERIAL AND METHODS

### Chemicals

Cumene hydroperoxide, 2,4-Dinitrophenylhydrazine (DNPH), and Guanidine-HCl were purchased from

Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest grade commercially available.

### Animals

All the procedures concerning animals were approved by Local Ethical Committee (LEC) and performed according to the instructions authorised by LEC. 50 male Syrian hamsters aged 4 weeks were divided into two groups. 40 hamsters (group 1) were implanted subcutaneously with 25 mg of 17- $\beta$ -oestradiol (Sigma-Aldrich Co E-8875). The other 10 animals (group 2) did not receive any treatment. After 1, 3, 6 and 9 months of experiment the animals were sacrificed and their kidneys were excised for further biochemical assays and histopathological evaluation. The weight of the testes was used as a marker of oestrogenisation effectivity and proved to be at least 12 times lower in each of the animals treated with oestrogen as compared to the controls.

All the animals were kept in the animal facility at room temperature, standard humidity and 12 h day/night circadian cycle. They were fed with standard chow and given water *ad libitum*. The animals were sacrificed and the kidneys were removed. A small part of each kidney was fixed with PA (4%). The rest of the kidney was homogenised.

### Preparation of homogenates

The kidneys from each animal were homogenised in 150 mM KCl, 10 mM Tris/HCl, pH 7.4, to form a 10% suspension.

### Carbonyl group assessment

This was performed as described by Oliver et al. [17] with modifications described by Kobiela et al. [7].

The sulphhydryl group assessment was performed according to the procedure described by Lou et al. [13].

### Histology

Multiple paraffin-embedded histological sections of 40 animals treated with E2 and 10 controls were stained with H&E and examined by light microscope. The animals were treated for 1, 3, 6 and 9 months.

### Statistical analysis

Each assessment was performed 3 times and as the data were not significantly different, the results were combined together. The analysis was performed using STATISTICA 6.0 PL and included ANOVA models and t-Student tests.

**Table 1.** Protein peroxidation. Levels of the carbonyl and sulphhydryl groups in the kidney tissue of the male Syrian hamsters treated with oestradiol for 1, 3, 6 and 9 months and in an untreated control group.

Group/Time of oestrogenisation		0 months	1 month	3 months	6 months	9 months	ANOVA
Carbonyl groups (nmol/mg of protein)	Oestradiol	—	3.30 ± 1.12	4.51 ± 0.77	5.02 ± 2.11	2.65 ± 0.79	p = 0.049
	Control	2.03 ± 0.4	1.86 ± 0.54	1.81 ± 0.31	1.77 ± 0.28	2.35 ± 0.27	p = 0.474
	t-Student	—	p < 0.01	p < 0.00	p = 0.02	p = 0.275	
Sulphydryl groups (nmol/mg of protein)	Oestradiol	—	66.7 ± 12.1	46.2 ± 8.23	34.5 ± 11.4	86.7 ± 31.1	p = 0.089
	Control	93.3 ± 19.3	99.1 ± 20.1	101.2 ± 22	97.2 ± 27.2	95.6 ± 33.9	p = 0.724
	t-Student	—	p < 0.01	p < 0.01	p < 0.01	p = 0.331	

Statistical significance in t-Student tests (between groups — in columns) in ANOVA (within groups — rows); data given as mean ± standard deviation in nmol/mg of protein). Note that oxidative stress is reflected by an increase in carbonyl groups and decrease in sulphhydryl groups

**Table 2.** Correlation of the biochemical markers of protein peroxidation with the most characteristic histopathological changes

Group/ Parameter	Level of carbonyl groups	Level of sulphhydryl groups	Most characteristic changes
1 month	↑	↓	Foci of proliferation
3 months	↑↑	↓↓	Tubular hyperplasia; interstitial hyperplasia
6 months	↑↑↑	↓↓↓	Tubular hyperplasia; interstitial hyperplasia
9 months	↑	↓	Tubular dysplasia; atypical stromal proliferation ( <i>Ca in situ</i> )
Control	—	—	No changes

## RESULTS

### Protein peroxidation

The intensity of protein peroxidative damage was presented in Table 1.

### Histological changes

Although the first discrete proliferation foci were observed as early as 1 month after treatment, they became significant after 3 months. Macroscopic tumours were observed after 9 months of the treatment. None of these lesions were detected in the control group (Table 2).

**1 month.** These early lesions, probably derived from the tubules, were the foci of crowded cells with hyperchromatic nuclei and eosinophilic cytoplasm (Fig. 1A).

**3 months.** Preneoplastic lesions at this point of the induction could be generally divided into those derived from interstitial cells (interstitial cell hyperplasia) and those of epithelial origin (tubular hyperplasia).

Hyperplastic proximal tubules were composed of clusters of cells with eosinophilic cytoplasm and generally uniform nuclei. However, in some cases they were irregularly arranged in layers that were 2 or more cells thick. The cells frequently involved the entire lining of the tubule and resulted in a micro-

cystic-like structure. Papillary-like epithelial formations were observed (Fig. 1B).

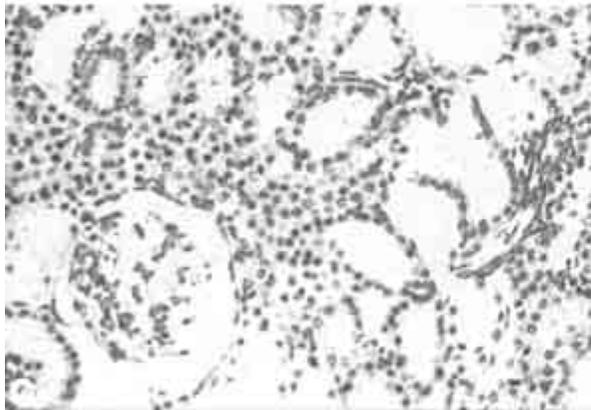
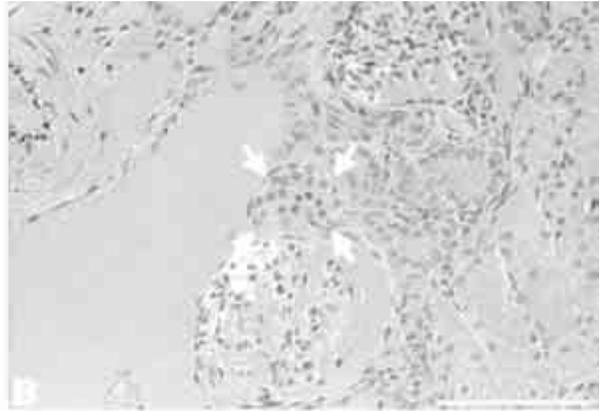
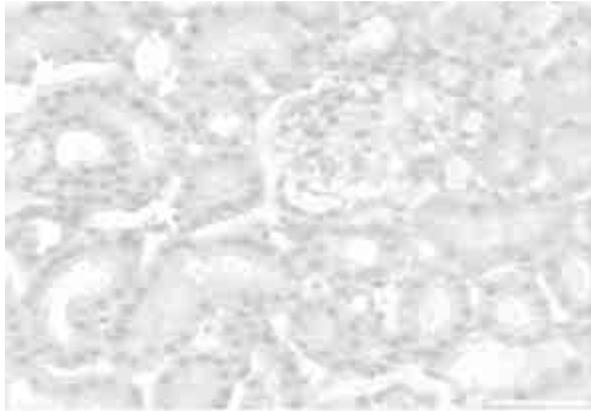
The hyperplastic foci of interstitial cells were often seen directly connected to the glomeruli. They were small, undifferentiated, basophilic, blastemal-type cells with dense chromatin (Fig. 1C).

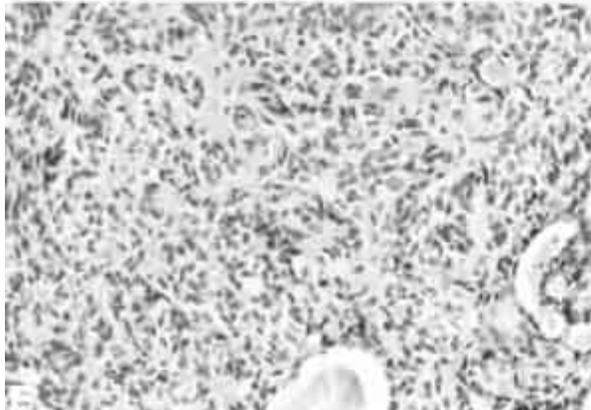
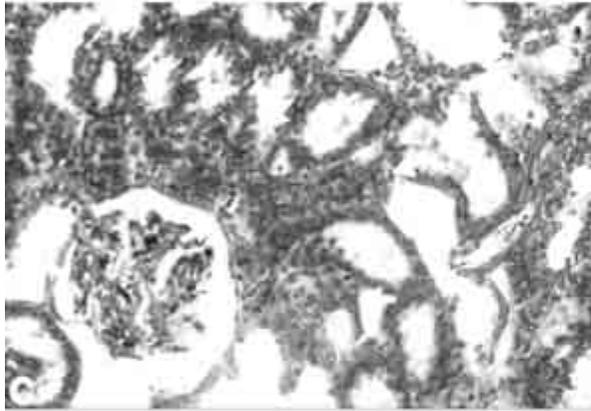
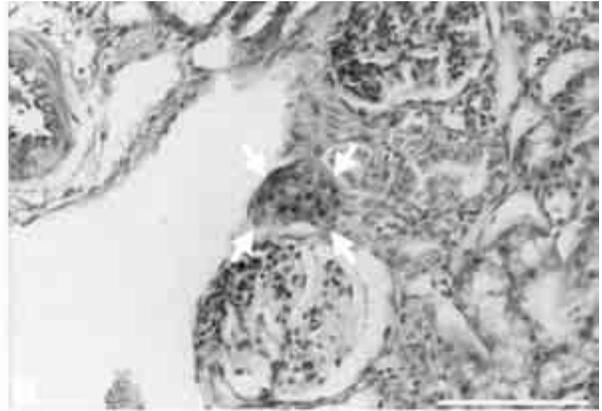
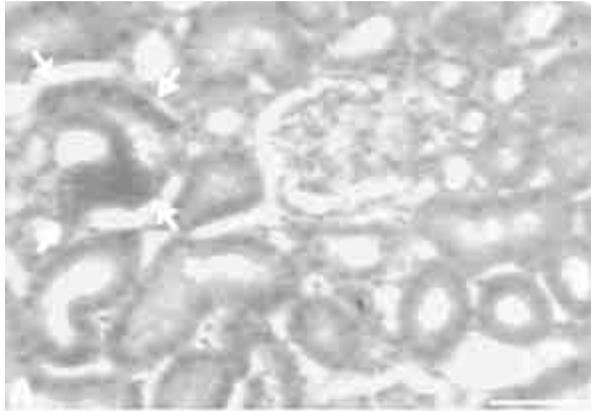
**6 months.** The histopathological picture of the kidney at this stage did not present evident progression of the lesions.

**9–10 months (including tumors).** A significant progression of the lesions was observed. Several tubular dysplasias forming microcysts layered with multilayer epithelial cells were observed. In some cases cilia were seen to arise from the luminal surfaces of the dysplastic cells. Some cells formed papillary formations inside the microcysts (Fig. 1D, F). The foci of carcinoma in situ were seen but not in progression to an invasive form. In parallel to this many areas of atypical stromal proliferation were found elsewhere (Fig. 1E).

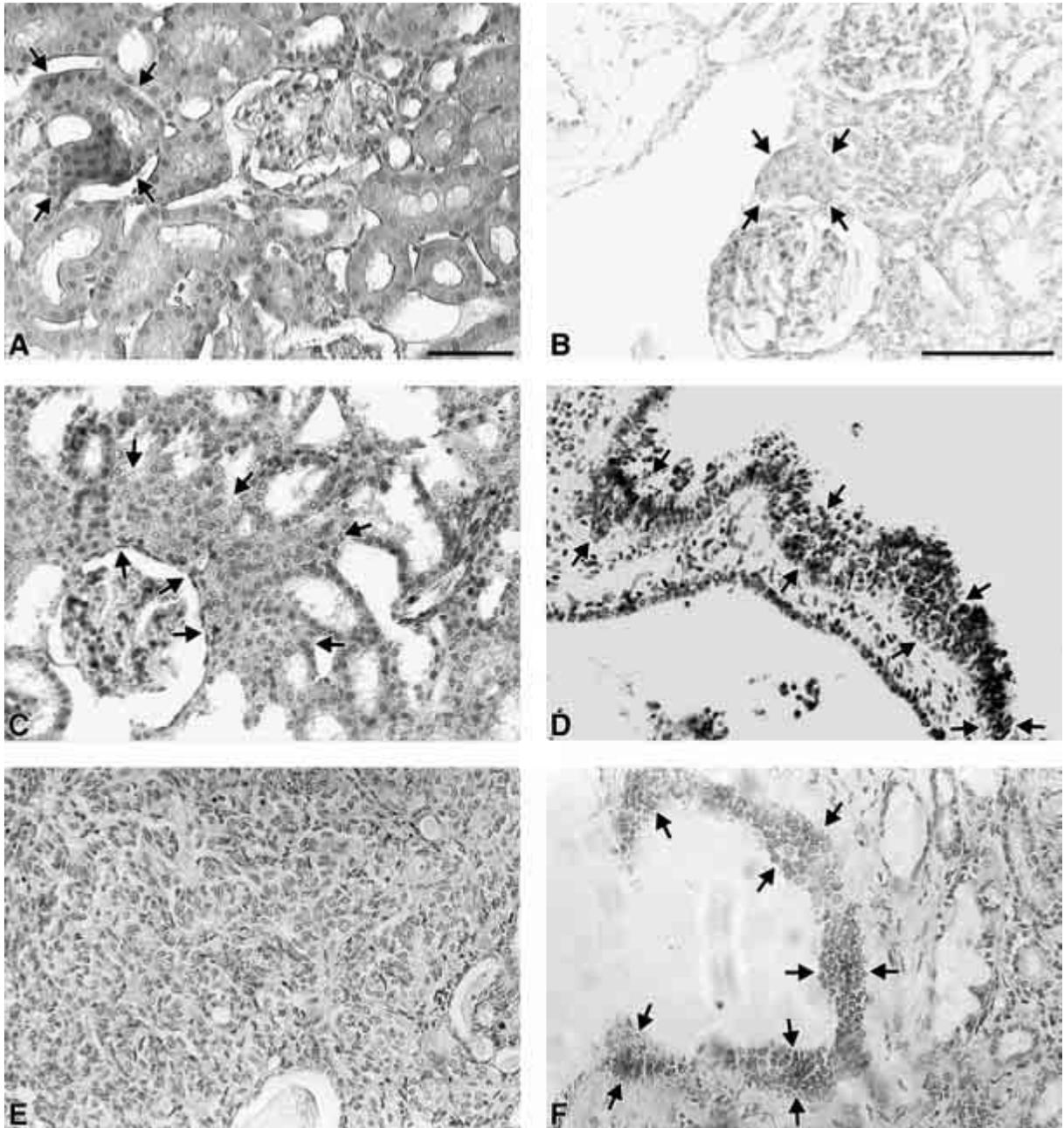
## DISCUSSION

The role of oxygen-free radicals and that of oestradiol-derived free radicals have been proved to be causal factors in kidney cancer in experimental male Syrian hamsters. The peroxidative activity of these radicals results in the destruction of proteins and









**Figure 1.** Changes in the kidney tissue of the male Syrian hamster (H & E); **A.** Cell proliferation after 1 month of oestradiol exposition (arrow); **B.** Tubular hyperplasia — 3 months treatment; **C.** Interstitial hyperplasia in the surroundings of the glomeruli — 3 months treatment; **D.** Advanced dysplasia: dysplastic cells inside the lining of the pathologic microcyst — 9 months treatment; **E.** Wide areas of small, dysplastic, blastemal-type interstitial cells — 9 months treatment (preinvasive cancer); **F.** Advanced dysplasia: abnormal pseudocyst formation with a multilayer lining of displastic cells and vivid disturbances of the architecture of the adjacent kidney tissue — 9 months treatment. Arrows indicate typical changes. Scale bar 100  $\mu$ m.

DNA [1], but not lipids. Although Liehr et al. [9] demonstrated a temporary increase in MDA level soon after the initiation of the carcinogenesis, their evidence was not strongly convincing. On the other hand, the protein peroxidation documented by an increase in carbonyl group level, a decrease in sulphhydryl group level and specific changes in the electron paramagnetic resonance (EPR) pattern have re-

cently been presented by Kobiela et al. [7]. Interestingly, protein stress was not accompanied by the peroxidation of lipids, measured by a more specific and reliable method, that is the level of lipid hydroperoxides. Moreover, EPR readings obtained after pre-incubation with 5-doxyI-stearinic acid did not reveal any significant change from the non-oestrogenised control group. It is worth mentioning that

apart from the lack of lipid peroxidation observed 1 month after the initiation of the oestrogenisation, no lipid peroxidation was noticed 1, 3 and 5 hours after the injection of the oestradiol (unpublished data). The predominance of protein peroxidation seems to present a sensitive and early marker of the oestrogen-dependent experimental kidney carcinogenesis [7].

One month of the oestradiol exposition was enough to detect protein peroxidation, measured both by the level of carbonyl as well as by sulphhydryl groups. At this point foci of proliferating cells were observed. Although the lesions could not be defined beyond doubt as preneoplastic lesions, they presented a high mitotic status different from the control group. These lesions can be described as resulting from oestradiol mitogenic activity, but can present groups of potentially mutated cells secondarily stimulated by oestrogen. The typical histopathological lesions, namely hyperplasias of tubular and interstitial origin, have been found to correlate with an increased level of carbonyl groups and a decreased level of sulphhydryl groups respectively after 3 months of treatment with oestradiol. Similar kinds of lesions have already been described by other authors [12], though these reports lacked relation to the biochemical changes. Although the protein peroxidation increased further after 6 months of the hormone exposition, no succeeding progression of the microscopic changes was observed. This phenomenon can be due to enzymatic burnout caused by extensive and prolonged oxidative stress. At this time severe dysplasias with a high grade of atypia were noted, sometimes leading to carcinoma in situ. Similarly, Cortes-Vizcaino and Llombart-Bosch [3] and Cortes-Vizcaino et al. [4] presented carcinoma in situ and several types of advanced cancer in animals treated with oestradiol for 9 to 12 months.

The increase in intensity and severity of preneoplastic and further early cancerous growth has been presented to show a strong positive relationship with the intensity of protein oxidative damage. Nevertheless, it should be underlined that the experimental model of oestrogen-dependent kidney cancer in the male Syrian hamster still poses multiple questions. The pathogenesis of the cancer, especially the multifocal role of oestradiol still calls for thorough study. The other problem is lies in inconsistencies over the origin of the cancer. The most accepted contemporary hypothesis suggests a stem cell origin of the cancer with further differentiation into interstitial or epithelial pathways [14]. On the basis of the study presented here, we strongly favour this theory.

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