



Proteasome: A Novel Potential Biomarker of Skin Cancer in Mouse Model

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Abstract

Background: The ubiquitin-proteasome system is an important regulator of cell growth differentiation and apoptosis. Recently proteasome levels have been suggested as a marker of various cancer diseases. In this study, we investigate the involvement of proteasome in skin cancer.

Methods: A mouse model of DMBA induced skin cancer was developed, papilloma or skin tumor confirmed by histopathological analysis. Proteasomes levels were measured using a sandwich ELISA test. The catalytic activity of the 20S proteasome was determined by measuring the fluorescence emitted after the cleavage of AMC.

Results: The results suggested that while all mice in the carcinogenesis group developed tumor-like forms of papilloma, control mice did not show any tumor formation. The histological skin analysis of carcinogenesis mice showed change, illustrated by hyperkeratosis, together with an irregular proliferation, and acanthosis in the epidermis. The serum proteasome and catalytic activity detected in the control group was much lower compared to those obtained in carcinogenesis group. Furthermore, we also noted that a higher level of intracellular proteasome concentration and an important catalytic activity were detected in the carcinogenesis group in comparison to control groups.

Conclusion: The proteasome may represent a novel marker of skin cancer. It could be a key element in the differentiation of normal cells from malignant cells, and may be useful in monitoring the clinical development of malignant cells.

Keywords: Proteasomes; DMBA-induced skin carcinogenesis; Chymotrypsin-like activity; Skin cancer

Abbreviations

UPS: The Ubiquitin-Proteasome System; ChT-like: Chymotrypsin-like activity; PGPH: Caspase-like; T-like: Trypsin-like; DMBA: 7,12-Dimethylbenz[a]anthracene

Introduction

Skin cancer is one of the most common malignancies affecting humans worldwide, and its incidence is rapidly increasing [1]. It develops in the epidermis and commonly of three types, basal cell carcinoma, squamous cell carcinoma, and melanoma [2]. One of the potent skin carcinogen is 7,12-Dimethylbenzene[a]Anthracene (DMBA) that is routinely employed in preclinical studies for understanding the pathogenesis of skin cancer and evaluation of novel therapeutic agents [3]. It is essential to search for a new biomarker of skin cancer at the very beginning of the disease. In spite of research aiming to uncover novel biomarkers linked to predicted aggressive skin cancer, attention has been called to only few of them, and their use in clinical practice has been recommended [4]. Examining the epidermis proteome in DBA/2 sensitive and C57BL/6-resistant mice upon the administration of TPA, 19 differentially expressed proteins were found. When examining the association between these 19 upregulated proteins, it was shown that they are active players in several inflammatory networks involved in skin tumor promotion, such as TNF- α and nuclear factor NF- κ B [5,6]. Proteomics still has an important input in the nearby future in this domain as its rapid evolving methodologies find their place in quantifying the proteome behind the subtle process of tumorigenesis [5,6].

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Normal growth and cell metabolism are not only dependent on the presence and activation of critical proteins but also on their degradation in time, allowing thus a successful cell cycle. A key player in the degradation of cell proteins is the proteasome [7]. Proteasome is a non-lysosomal proteolytic structure localized both in the cytoplasm and in the nucleus of eukaryotic cells that have been implicated in many important biological processes, including cell growth, cell differentiation and apoptosis, such as cyclins and CDK, the proapoptotic protein p53, or NF-kappa B of normal as well as tumor cells [7,8]. It corresponds to major system-degrading abnormal proteins, short half-life proteins, and proteins that control the cell cycle [8,9]. The 20S proteasome has been found as an isolated complex associated with one or two regulatory particles, the 19S subunit or 11S subunit of the regulatory complex that recognizes poly-ubiquitinated proteins, unfolds them, and passes them into the 20S catalytic Core Particle (CP) for degradation [10,11]. The barrel-shaped 20S is comprised of four -stacked and seven-membered rings, two inner β rings, and two outer α rings. The α rings provide a contact point for 19S regulators or for the PA28 proteasome activator, forming thus 26S proteasomes (19S-20S), 30S proteasomes (19S-20S-19S), PA28-20S proteasome-complexes or hybrid proteasomes (PA28-20S-19S), respectively [11]. The β rings contain three main proteolytically active subunits with β 1 exhibiting caspase-like, β 2 trypsin-like and β 5 Chymotrypsin-like (ChT-like) activity through which proteasomes degrade proteins [12,13].

Many studies have demonstrated the involvement of the proteasome in various types of cancer diseases like multiple myeloma [14], hepatocellular carcinoma [15], and epithelial ovarian cancer [16]. Several works on this subject have focused on proteasome inhibition as a new strategy for cancer therapy; the knowledge on the proteasome for disease diagnosis is insufficient. In 2005, Stoebner et al. [17], reported that in 20 patients including those with breast, gastric, kidney, colon, testicular, liver and lung cancer, the proteasome 20S serum level was significantly elevated compared with controls, indicating that serum proteasome could be applied in tumor diagnostics.

Furthermore, Plasmatic Proteasome is a potential marker in patients with hemopoietic malignancies like acute leukemia, myeloproliferative, myelodysplastic, syndromes, chronic lymphocytic leukemia, non-Hodgkin lymphoma and Hodgkin disease [18]. In 2010, Henry et al. [19], suggest in a study carried on 90 patients with metastatic melanoma and 40 controls that the plasmatic proteasome level is a potential diagnostic biomarker of Human melanoma.

Additional studies using a xenograft animal model system in which C8161 melanoma cells were injected suggest that the inhibition of the proteasome can mediate apoptosis in melanoma cells [20]. It has been demonstrated that the inhibition of proteasome induces cell death more strongly in neoplastic cells than in normal cells, and that, in addition, proteasome inhibition sensitizes neoplastic cells to other proapoptotic stimulus such as chemo or radiation therapy. Therefore, the proteasome could be a good target for cells that are as resistant to apoptosis as melanoma and non-melanoma cells [19,21]. In spite of these data, there is little information about the implication of the proteasome in cutaneous tumors *in vivo*.

In this work, proteasome levels and chymotrypsin-like activity were analyzed in mice developing skin cancer, the aim being to evaluate the possibility of integrating proteasome as a biomarker for skin cancer.

Materials

Reagents

7,12-Dimethylbenz[a]Anthracene (DMBA, (D3254)), Croton oil (C6719) and substrate Suc-LLVY-AMC (chymotrypsin-like activity) were purchased from Sigma-Aldrich. Primary Monoclonal antibody MCP20 for the α 6 subunit and the polyclonal proteasome 20S core antibody (PW 8155) subunits were obtained from (Enzo Life Sciences). Anti-rabbit IgG Antibody (HRP) (Cat. No.252237) from (ABBIOTEC, San Diego, CA92126, USA). Goat Anti-rabbit IgG (H+L) Mouse/Human (ads-AF488) from (SouthernBiotech, Birmingham, USA). All other used chemicals were of analytical grade, purchased from Sigma-Aldrich.

Animals

Adult female Swiss albino mice, 6 to 8 weeks old (23.5 ± 2 g) were obtained from the pet shop of our institute, and were housed in polypropylene cages. The animals were acclimatized for 1 week prior to the start of the experiment. Mice were fed with commercially available Food pellets and water ad libitum, and maintained on standard housing conditions under controlled atmosphere with 12:12 h light/dark cycles, with an ambient temperature of 25 ± 5 C and humidity at $50\% \pm 10\%$. Animal handling and experimental protocol were conducted according to the guidelines of the Institutional Ethical Committee.

Two-stage chemical carcinogenesis

To induce carcinogenesis in mice, approximately 2.5 cm² of dorsal body wall hair was removed by shaving with electric clippers, followed by application of depilatory cream one day prior to the beginning of the experiment. Then mice were initiated with single topical application of DMBA in acetone (100 μ g/100 μ l) on shaved dorsal skin. A week later, this was followed by two topical weekly applications of 1% croton oil in acetone (1 μ l/100 μ l; promoter) at the same site for 20 week. The Normal Control (NC) mice received only acetone in place of DMBA and croton oil [3] Table 1.

Morphologic observation of papilloma development

During the 20 weeks of experiments, mice were observed daily for the appearance of skin papillomas tumor volume, and body weights were recorded at an interval of 7 days until sacrifice. Blinded to experimental groups, an observer counted, measured, and scored tumors as clinically apparent papillomas (well demarcated, symmetrical, pedunculated, or dome-shaped papules, without erosion or ulceration).

Sample collection and tissue preparation for histology

Animals were killed at the end of the experimental period by cervical decapitation. Blood was collected and serum was separated for analysis, using a sandwich Enzyme-Linked Immunosorbent Assay (ELISA). Skin tissues (tumors and normal skin) were excised from the animals and fixed in 10% neutral formalin for histopathological studies.

Cell lysis

The serum is recovered from a mouse blood sample and gradually cooled down from 4°C to 20°C. The lymphocyte pellet is recovered by the lysis of red blood cells.

First we proceed to the preparation of the white cell pellet. Briefly, we break the red cells of the blood sample by adding a solution of Tris-EDTA (20/5) to the whole blood; after centrifugation, the

Table 1: Experimental groups of mice.

Groups	Treatments
Normal control group (NC)	Female Swiss mice received acetone application in the previously shaved dorsal region for 20 weeks
Carcinogenesis group (CC)	Female Swiss mice were shaved dorsally for application, once in the first week (initiation phase); the application of the DMBA solution (DMBA dissolved in acetone) was applied to the shaved backs of the animals. After that, two applications of the croton oil were applied in the same area of the shaved backs of the mice during 20 weeks (promotion phase)

supernatant is removed and we repeat this procedure until we obtain a clear cell layer. We then rapidly add 200 μ l of lysis buffer (10 mM KCl, 10 mM NaCl, 10 mM HEPES, 1 mM EDTA pH 7.1, 0.1 mM DTT, 1% Triton, supplemented with protease inhibitors (PMSF 2 nM) on each sample. The heating of cells is disrupted during breaks of 20s to 30s to avoid the excessive heating of samples, which can cause denaturation of enzymes [22]. The protocol is repeated 3 times. The extracts obtained may be stored at -20°C until their use.

20S Proteasome quantification by ELISA assay

The sandwich ELISA used here is a combination of a monoclonal antibody (Ab MCP20 that recognized only the $\alpha 6$ subunit), and a polyclonal rabbit anti-20S antibody (PW 8155), which labeled different subunits of the 20S complex. ELISA assays were realized in 96-well plates. First, the plate was coated with 100 μ l of Ab MCP20 monoclonal antibody at 5 g/ml at 37°C over 1h. Wells were then washed three times with PBS/T-buffer. Nonspecific sites were blocked by incubation with 200 μ l of PBS- BSA 2% for 1 h at room temperature with slow shaking. The plate was washed three times with PBS/T-buffer. 100 μ l per well of the samples were then deposited in duplicate and incubated for 2 h at room temperature. The plate was washed with PBS/T-buffer, and then incubated with the polyclonal 20S antibody for 1 h at room temperature under slow shaking. The plate was washed three times with PBS/T-buffer, and antibody binding was detected by using horseradish peroxidase conjugated, anti-rabbit antibody, and OPD (ortho-phenylenediamine). The reaction was monitored by measurement of the optical density at 492 nm using an ELISA reader (ELx800 UV, USA). The concentration of proteasome in the samples is expressed in ng/mL, after comparison with the calibration curve obtained with the standard purified 20S proteasome from human erythrocytes [19].

Measurement of proteasome activity

As described previously [22], the catalytic activity of the 20S proteasome on the fluorogenic peptide Leu-Leu-Succinyl-Val-Tyr-Amido-4-methylcoumarin (Suc-LLVY-AMC) was determined after

substrate incubation with the sample at 37°C over 3 h, by measuring the fluorescence emitted after cleavage of AMC, using a fluorometer (Heofer Scientific Instruments). The excitement of peptide coupled to the AMC is done through a filter 360/40 nm filter whose that the wavelength is between 340 nm and 380 nm at maximum 360 nm. The fluorescence emitted after digestion of peptides coupled to the AMC is read on the 460/40 nm filter. To convert the Unit of Fluorescence (UF) issued in μ mol of used fluorophores, a standard AMC range is established from a stock solution of AMC at 10 m to 3 m. The excitement of AMC is done at a wavelength of 360/40 nm and the signal is then recovered on the 460/40 nm filters.

Histopathological evaluation

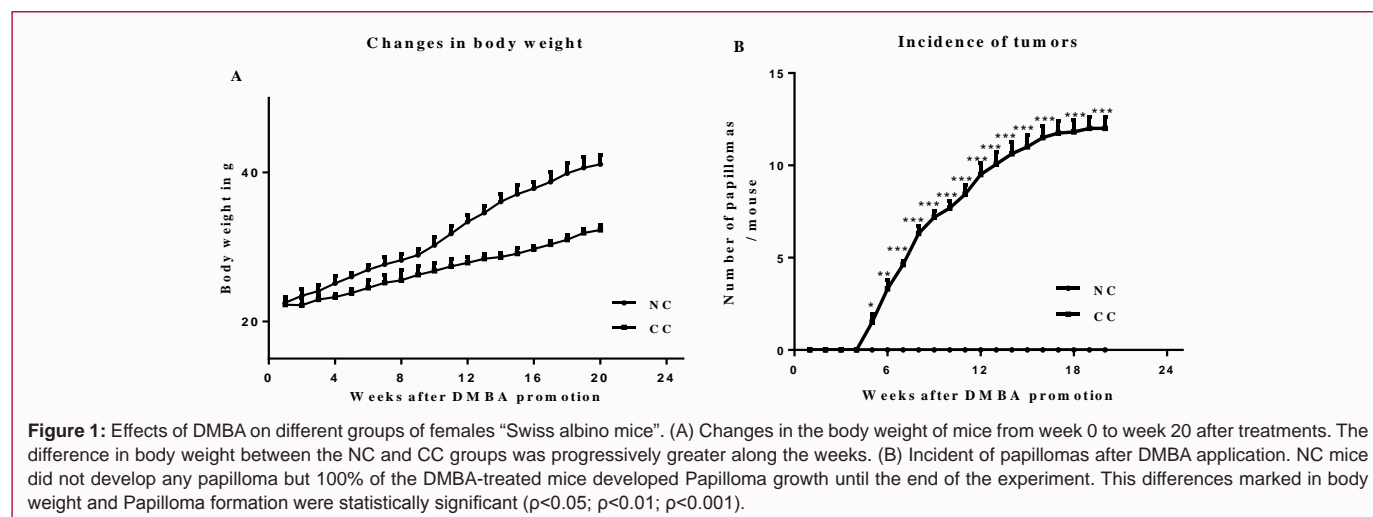
Collected skin tumors were fixed in 10% neutral formalin for 24 h and then passed through ascending grades of ethyl alcohol starting from 60% to 95%. The dehydrated tissues were then soaked in toluene and then transferred to molten paraffin (60°C), which was poured in metal moulds; the tissues were set accordingly. Serial microtome sections in the form of a paraffin ribbon were made at a thickness of 4 μ m. To stretch the tissues, the latter were then floated in a tray containing lukewarm water (58°C). Tissue sections (3 μ m to 4 μ m) were taken for Hematoxylin-Eosin (HE) staining and histopathological evaluation.

LDH measurement

LDH was assayed by measuring the rate of oxidation of NADH at 340 nm according to the method of Bergmeyer and Berntas reported [24]. The enzyme activity was calculated using an extinction coefficient of 6.22/mM/cm. One unit of enzyme activity was defined as that causing oxidation of 1 mol of NADH/min.

Statistical analysis

All data obtained are represented as the mean \pm Standard Error of the Mean (SEM). Student's t-test was used to test whether differences between two groups were significant. Analyses were performed using GraphPad prism 6 software. A p values <0.05 were considered



significant.

Results

Body weight loss

We noticed that the body weight of Normal Control mice (NC) showed a normal increase of ($22.58 \text{ g} \pm 0.79 \text{ g}$) to ($41.87 \text{ g} \pm 1.45 \text{ g}$) at the end of the experiment. Whereas, the evolution of body weight in the Carcinogenesis mice (CC) was much lower than that of normal mice control; furthermore, the average body weight at the end of the experiment was ($35.6 \text{ g} \pm 1.07 \text{ g}$).

There was a sharp difference in body weight noted between the (NC) and (CC) groups at the end of this study. The difference in body weight between the NC and CC groups was progressively greater along the weeks (Figure 1A).

Skin papilloma development

The tumor incidence in both normal control mice and carcinogenesis mice was recorded. We observed that tumor appearance, and development in the carcinogenesis group started in the 5th week after DMBA and croton oil applications (Figure 1B). Furthermore, we remarked that all mice in the carcinogenesis group had developed a tumor like papilloma form at average number ($9.6 \pm 3.86/\text{mouse}$) (Table 2, Figure 2B, 2C). Tumors increased progressively in number and size throughout the study period. On the contrary, control mice did not show any damage or tumor formation (Figure 2A). This noticeable difference was statistically significant (Table 2). In addition, the results suggested that carcinogen mice developed tumors as papillomas with a diameter of 1 mm to 8 mm and height of 1 mm to 5 mm. More so, following the first application of DMBA, we noted the appearance of lesions, damage and severe skin inflammation. However, Control mice did not demonstrate any papilloma formation or lesion apparition due to acetone application alone during the experiment (Figure 2A).

Histology of skin after induction of carcinogenesis

Normal skin is composed of two main layers: The outer epidermis, consisting of squamous epithelial cells, and the inner dermis, which consists of connective tissue. The epidermis, which is composed of several layers of squamous epithelium, shows the thickness and distribution of the cells of normal epidermis [25]. The dermis (the underlying layer of the epidermis) consists of two layers: The papillary layer, consisting of the loose connective tissue, and the reticular layer, with dense connective tissue. These layers are so closely related that they are difficult to differentiate. However, after repetitive exposures to carcinogens agents, an undifferentiated layer takes on the papilloma growth characteristics, ultimately leading to tumor formation [25].

The histological sections of skin from control mice (NC) showed a normal cell distribution in the different layers making up the skin, as neither hyperplasia nor irregular cell proliferation was observed (Figure 3A). Whereas, histological skin analysis of carcinogenesis mice (treated with DMBA/croton oil) showed atypical change of skin illustrated by hyperkeratosis and a canthus is in the epidermis (Figure 3B, 3C). Furthermore, observed skin cross-sections revealed hyperkeratosis, dysplastic lesion, acanthosis with an irregular cell proliferation in the stratum spinosum, and papillomatosis of the epidermis (Figure 3B). In addition, those histological sections of skin or papillomas revealed hyperplasia resulting in a thickening of the epithelium and Papilloma without atypia. Moreover, a moderate to severe hyperplasia, followed by the formation of papillomas, hyperkeratosis, increased melanocytes, spongiosis and horny cysts were seen. Papillomas progressed to squamous cell carcinoma and showed atypical marked changes (Figure 3C, Table 3).

Proteasome measurement

Proteasome levels in control mice and carcinogenesis mice: The serum and intracellular levels of proteasome were ($1505.11 \pm 81.31 \text{ ng/mL}$; $1945 \pm 119.55 \text{ ng/mL}$) respectively for the normal control

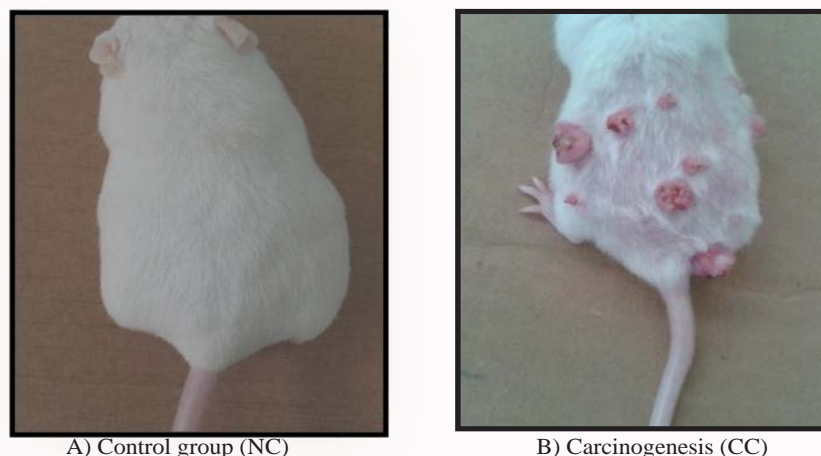


Figure 2: Effects of DMBA and croton oil on skin tumor formation in "Swiss albino mice": (A) Normal control mice treated with 100 μl of acetone alone in the dorsal area for 20 weeks, (B) Mice treated with one topical applications of 100 μg DMBA in 100 ml of acetone, followed by 100 μl of 1% w/v croton oil in acetone at the same site twice weekly until the end of the experiment.

Table 2: Characteristics of experimental groups.

Experimental group	No. of animals/duration of study (weeks)	Initial average weight (g)	Final average weight (g)	% of mice with papillomas	Average No. of papillomas/mouse
Control group	20/20	22.58 ± 0.79	41.87 ± 1.45	0	0
Carcinogenesis group	20/20	22.4 ± 0.63	35.6 ± 1.07 ($p < 0.05$)	100% ($p < 0.001$)	9.6 ± 3.86 ($p < 0.001$)

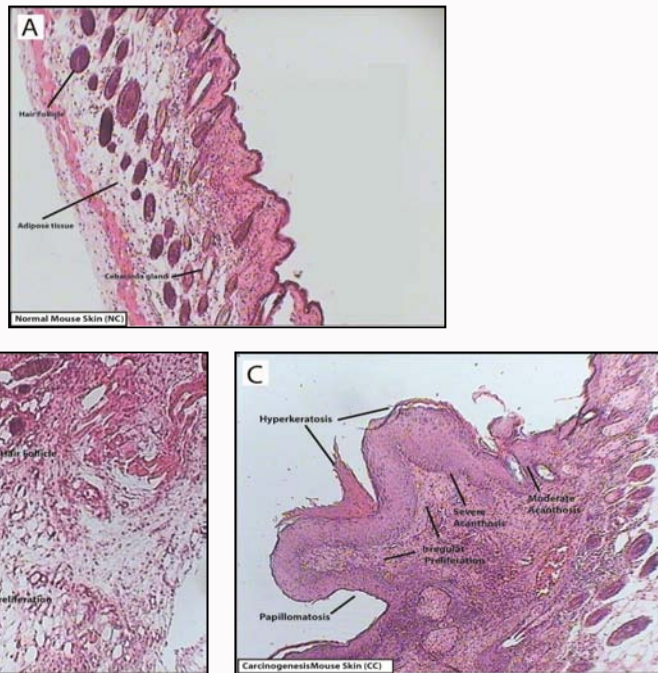


Figure 3: H&E stained cross-sections of mouse skin: (A) normal mouse skin (NC) showing (B) carcinogenesis mouse skin (CC) after 12 weeks of exposure to DMBA/croton oil shows hyperkeratosis, moderate acanthosis with mild dysplasia (formation of abnormal cells) and papillomatosis of the epidermis. (C) CC group after 20 weeks of exposure reveals marked papillomatosis, epithelial vascular lesion, and epidermal hyperplasia with hyperkeratosis, parakeratosis, spongiosis and marked acanthosis.

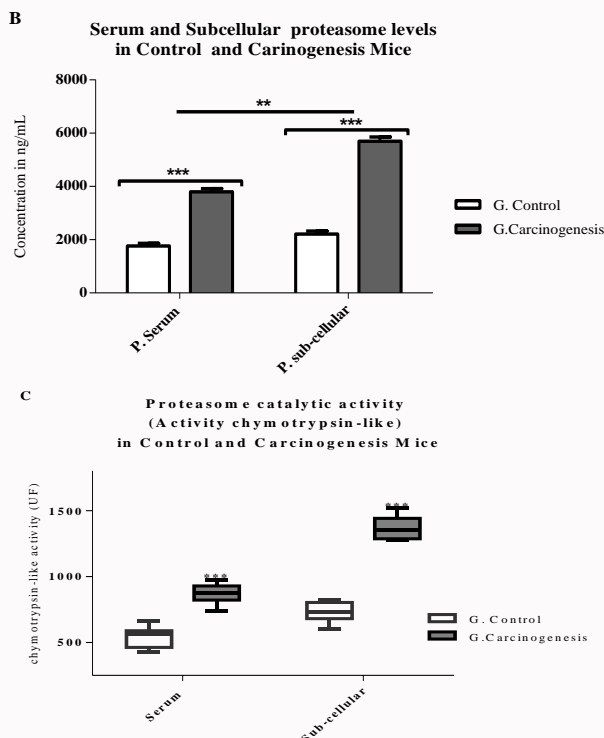


Figure 4: Measurement of Proteasome levels and catalytic activity of proteasome in controls and carcinogenesis mice. B) Serum and Subcellular proteasome levels in controls and carcinogenesis mice. Serum and subcellular proteasome level was higher in carcinogenesis mice (3027.44 ± 121.36 ng/mL; 4702.22 ± 109.10 ng/mL, $p < 0.001$) compared with control group (1505.11 ± 81.31 ng/mL; 1945 ± 119.55 ng/mL). C) Evaluation of the proteasome catalytic activity (chymotrypsin-like activity) in control and carcinogenesis groups. The results shown a significant activity was measured both in serum.

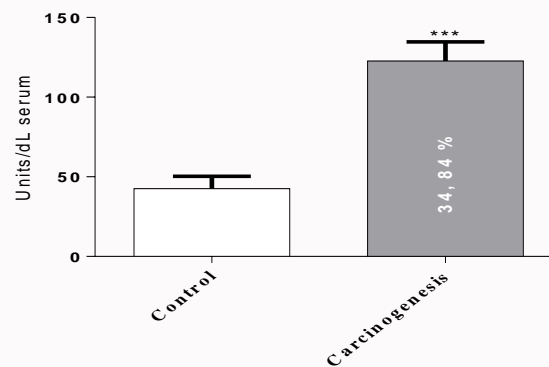


Figure 5: Lactic dehydrogenase activity in the serum of control and carcinogenesis mice.

group and (3027.44 ± 121.36 ng/mL; 4702.22 ± 109.10 ng/mL) for the carcinogenesis group respectively. According to those results, we noted that the serum proteasome concentration in the control group was much lower compared to those obtained in the carcinogenesis group (Figure 4B). Furthermore, we noted a higher level of intracellular proteasome in the carcinogenesis group compared to the control group (Figure 4B). This observed difference between Proteasome levels both in control mice and in carcinogenesis mice was statistically significant ($p < 0.001$).

Chymotrypsin-like activity of proteasome: The evaluation of catalytic activity (chymotrypsin-like activity) of proteasomes in control and carcinogenesis mice was measured as described previously [28]. The results shown in Figure 4C suggest, that in the serum, the catalytic activity (878.87 ± 26.150 UF) was significantly ($p < 0.001$) higher in the carcinogenesis group compared to the control group (503.43 ± 12.45 UF). Likewise, the average catalytic activity

Table 3: Mice percentages in two experimental groups, showing different histopathological stages.

Stages	Percentage of mice showing this stage	
	Control group	Carcinogenesis group
Squamous cell carcinoma	0/20 (0%)	20/20 (100%)
Dysplastic lesion	—	20/20 (100%)
Papilloma without atypia	—	20/20 (100%)
Papilloma with hyperplasia	—	20/20 (100%)

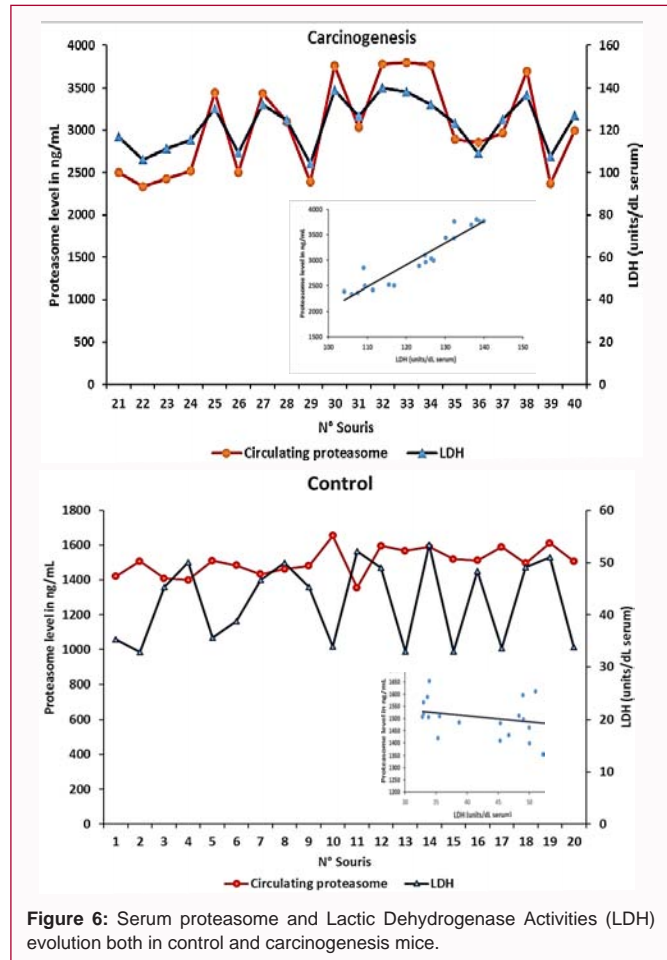


Figure 6: Serum proteasome and Lactic Dehydrogenase Activities (LDH) evolution both in control and carcinogenesis mice.

assayed for the intracellular proteasome (1368.75 ± 87.28 UF) was significantly ($p < 0.001$) higher in the carcinogenesis group than that measured in the control group (732.25 ± 77.08 UF).

LDH measurement: The measurement of LDH was given in Figure 5. We have noted that LDH levels in control mice was much lower with on an average of (42.506 ± 7.84) in comparison with the one measured in carcinogenesis; the latter had important LDH levels (122.7125 ± 11.93 ; $p < 0.001$). In addition, we have noted that LDH activity was increased by about 34.84% with tumor development.

Correlation between proteasome levels, chymotrypsin-like activity and Lactic Dehydrogenase (LDH):

a) Comparison between proteasomes levels and LDH activities: We have noted that there is no correlation between serum proteasome levels and LDH activity in control mice (Figure 6). On the contrary, the results obtained in carcinogenesis mice showed a high correlation ($R=0.95$; $P < 0.0001$) between serum proteasomes and measured LDH

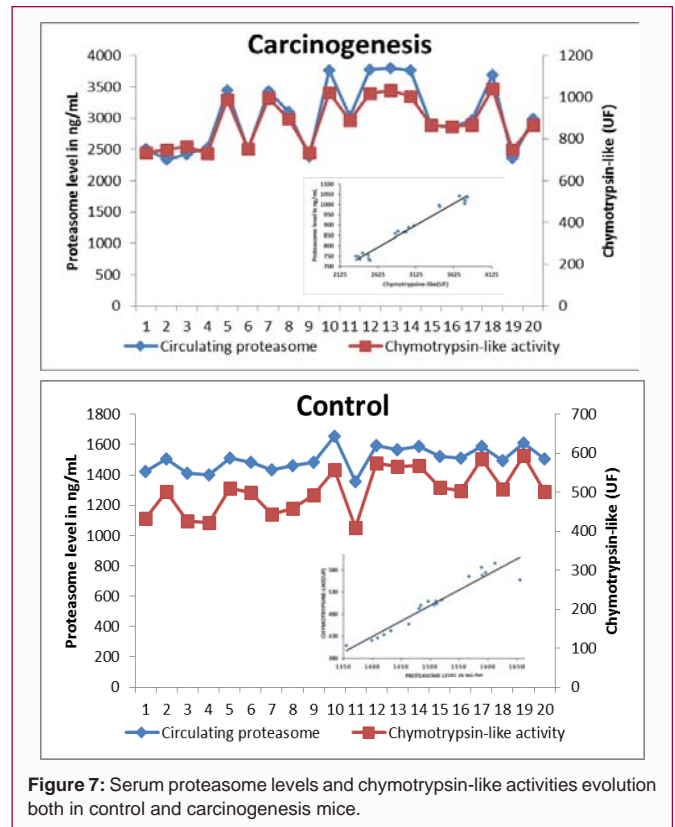


Figure 7: Serum proteasome levels and chymotrypsin-like activities evolution both in control and carcinogenesis mice.

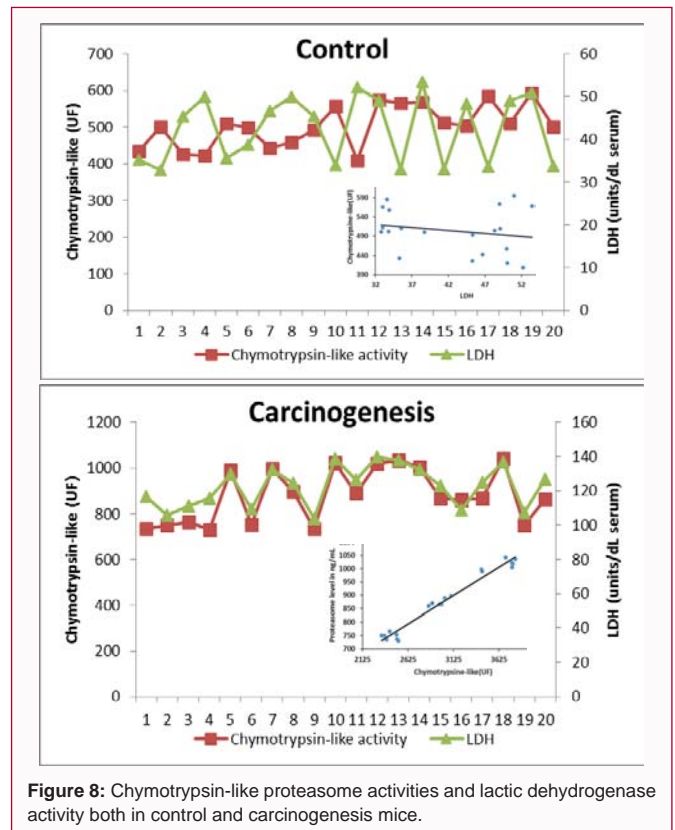


Figure 8: Chymotrypsin-like proteasome activities and lactic dehydrogenase activity both in control and carcinogenesis mice.

activity Furthermore, we have noted that both proteasome levels and LDH increase with tumor development.

b) Comparison between proteasomes levels and chymotrypsin-

like activity: The evolution of the correlation between serum proteasome levels and chymotrypsin-like activity both in control and carcinogenesis mice was given in Figure 7. The results obtained demonstrate that there is a correlation ($R=0.96$; $P<0.0001$) between serum proteasome levels and chymotrypsin-like activity measured in control mice. Moreover, an important correlation ($R=0.98$; $P<0.0001$) was observed in carcinogenesis mice.

c) Comparison between chymotrypsin-like and LDH activities: As shown in Figure 8, the results of chymotrypsin like and LDH activities suggest that there is no correlation in control mice. Furthermore, an important correlation ($R=0.92$; $P<0.0001$) was observed between chymotrypsin-like and LDH activities in carcinogenesis mice.

Discussion

The transformation of normal cells to the malignant state proceeds through several discernible stages, including initiation by DNA damage and later events that have been defined as tumor promotion in animals and *in vitro* [26]. The objective of this study was to investigate the impact of the proteasome on the development of melanoma in mouse the model induced by DMBA, as well as the catalytic activity on both serum and intracellular levels in different groups of mice (control mice and carcinogenesis mice). A comparison was made with LDH, which was considered to be a potential biomarker for advanced skin cancer courses.

The two-stage skin carcinogenesis model in mice offers a good opportunity to study the mechanisms of carcinogenesis from promotion, progression, and angiogenesis to metastasis. In this work, we use this model to investigate the role and the involvement of the proteasomes in melanoma development. In the current study, we have demonstrated that the topical application of DMBA and croton oil induce weight loss (Figure 1A), and tumor development. 100% of mice in the carcinogenesis group have developed a tumor-like form of papilloma in comparison with control mice (Figure 2). This tumor increases progressively in number and size throughout the period of the experience (Figure 1B). Our results are in agreement with numerous studies [3,26]. In fact, the application of DMBA and croton oil causes inflammation and oxidative stress-related DNA damage in skin that might lead to skin papilloma in female Swiss albino mice, through the formation of a DNA/DMBA adduct with genomic DNA [26], causing the expression of LPO and ROS in the affected area of the skin and leading ultimately to carcinogenesis [27-29]. The histological study of papilloma tissue showed numerous modifications in comparison to the normal tissue (Figure 3). Indeed, this was evident from the histological study of the skin samples taken from the carcinogenesis mice, an irregular cell proliferation, as well as epithelial vascular lesion, epidermal hyperplasia with hyperkeratosis, parakeratosis, spongiosis, marked acanthosis, dysplasia and papilloma growth [3,34]. Microvessel formation in papillomatosis was also observed, suggesting that the topical application of DMBA/croton oil caused tissue damage, angiogenesis and progression of tumors according to the literature [3,29].

The Proteasomes are highly organized structures that are responsible for a major part of non-lysosomal intracellular proteolysis [9]. The Proteasomes play a central role in the regulation of the cell cycle and various cellular processes, including proliferation, differentiation, apoptosis, and response to extracellular stimuli. The alteration of one of these major processes can lead to the development of carcinogenic mechanisms [9,10].

The anomalies in the ubiquitin-proteasome pathway have been described in various pathologic settings, including malignant disease. In this field, recent studies have shown that proteasome inhibition can induce tumor regression in animal models [30,31]. The proapoptotic effect has been validated in several clinical trials with various proteasome inhibitors [32]. The serum proteasome can be measured by the enzyme-linked immunosorbent assay technique (ELISA) [33]. We have applied such a technique to the measurement of serum and intracellular proteasome levels in different groups of mice. Although proteasome is mainly localized in cytoplasm and in the nucleus of eukaryotic cells, our results confirm the presence of proteasome in serum in all groups of mice (Figure 4). Furthermore, a higher level of intracellular proteasome was detected in comparison to serum proteasome; this can be explained by the production and synthesis of the complex in question in the interior of the nucleus, against low concentrations detected in serum, which can be due to cell lysis [20], or can be explained by the export of functional proteasome from activated immune cells by way of microparticles, the dissolution of which may finally lead to the generation of extracellular proteasomes [34]. The second part of our study concerns the impact of proteasome in skin cancer development. Indeed, our results have shown that serum proteasome levels are significantly elevated in carcinogenesis mice (2759.44 ± 71.73 ng/mL; $p<0.001$), against control mice (1130.11 ± 81.31 ng/mL). Like the serum proteasome level, a higher intracellular level has been detected in carcinogenesis mice (4702.22 ± 109.1 ng/mL; $p<0.001$), compared to control mice (1845 ± 119.55 ng/mL). Our data are in agreement with the ones obtained by Henry and their collaborators in a study of patients with metastatic melanoma [18]. Also, these results are consistent with the study of Lavabre-Bertrand [17], which was carried out on patients with solid tumors, hemopoietic malignancies, as well as with previous data reports from Moroccan patients with hematological malignancies [22]. The origin of higher levels of proteasomes in carcinogenesis mice detected both in serum and intracellular tracts the interest of several researchers; yet, it is still unclear. Papilloma cells may produce Proteasome, and could be due to the increase of poorly folded proteins generated by the metabolism of cancer cells [19]. During cell proliferation, a high traffic volume of proteasomes and proteasomal substrates arises between the cytoplasm and the nucleoplasm [35].

In addition, numerous studies suggest that the DNA/DMBA adduct promotes RAS and p53 mutations [29,36]. This mutation causes the activation of the proto-oncogene RAS which often code for proteins that stimulate cell division, avoid cell differentiation or regulate programmed cell death (apoptosis). The inactivation of P53 plays a crucial role in the majority of cancer diseases, particularly in the promotion and progression of skin carcinogenesis, inducing cell proliferation and angiogenesis [37].

In fact, the concentration of p53 in the cell is subjected to strong control by MDM2. Indeed, MDM2 is a phospho-protein that plays the role of ubiquitin ligase for P53; it allows among other things the export of P53 from the nucleus to the cytoplasm and its ubiquitination, thus promoting its degradation by the proteasome 26S [37,38].

Numerous studies have described the proteasome endows of tree catalytic activities as trypsin-like, chymotrypsin-like and PGPH-like [39]. The orientation assay of the chymotrypsin-like activity of the proteasome takes account of the fundamental role of this activity in the cycle of proteolytic degradation. Indeed, literary data confirms that the inhibition of chymotrypsin-like activity is sufficient enough

to allow a significant reduction in the rate of protein degradation. Whereas, the inactivation of other trypsin and PGPH-like sites has little effect on the total proteolysis [40].

Our data suggest that the chymotrypsin-like activity of proteasome in serum is higher in carcinogenesis mice than in control mice. Likewise, in intracellular, we report that the chymotrypsin-like activity of proteasome is more important in carcinogenesis mice. Hence, we emphasize that although LDH is considered a metastatic biomarker of melanoma, its proportional increase (34%) is relatively lower than that obtained for the circulating proteasome level (49%) and the catalytic proteasome activity (57%). Moreover, we noted a positive correlation between LDH, circulating proteasome, and catalytic activity, which is magnified only in mice developing cutaneous tumors. All data can reinforce the confirmation listed in the bibliography which considered that circulating proteasome is an eventual biomarker of skin cancer. There is need for other experiments to determine the degree of effectiveness of proteasome levels and catalytic activity as biomarkers at different stages of the disease. Our data are compatible with Xinghua Wei's findings [41], which suggest that Chymotrypsin like proteasomal activity is elevated by 70% *in vitro* and 23% *in vivo*. In addition, in comparison with normal mouse prostate tissue, the expression levels of the proteasome substrate proteins I κ B α , Bax and p27 are reduced in prostate cancer cells and in prostate tumor xenografts. In addition, previous data found in patients with leukemia confirm these findings [39].

It has recently been demonstrated that protein degradation by the proteasome is a biological process that is essential for the survival of cancer cells. Increased proteasome activity in tumor cells provides a certain resistance to apoptosis [42,43]. Apoptosis is a feature common to most malignant tumors. It is currently established that treatment with proteasome inhibitors provides favorable results in the treatment of certain liquid and solid tumors, especially melanoma [18-20].

The proteasome appears to be a key element of neoplastic differentiation. Quantitative and especially qualitative variations seem to play an important pathophysiological role. It seems to be an original and sensitive blood tumors marker. To reduce side effects and to improve patients' conditions, we propose to adjust the dose of treatment with proteasome inhibitors, on grounds of an estimated concentration and catalytic activity on the cell proteasome level.

Data Availability

All data generated or analyzed during this study are included in this published article.

Authors Contribution

LAHMADI Ayoub Conveived the study. Lahmadi Ayoub, Filali Hassan and Aboudkhil Souad designed all experiments. EL Anteri Said and Lazar Said participated in the biochemistry experiments. Lahmadi Ayoub, Aboudkhil Souad and Samaki Hamid performed the data analysis. Lahmadi Ayoub drafted and Aboudkhil Souad edited the initial manuscript. All authors read and approved the final manuscript.

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