MDM2 gene amplification in skin cancer patients

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دراسة تضخم الجين MDM2 في مرضى سرطان الجلد

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وحدة المناعة السريرية , والمناعة النسيجية الكيميائية , مدينة طب الأماميين الكاظميين (ع) الطبية , بغداد , العراق

المستخلص

سرطان الجلد: سرطان الجلد هو نمو غير طبيعي لخلايا الجلد غير المسيطر عليه , ويصنف إلى نوعين رئيسين هما غير الميلانوما (سرطان خلايا الجلد الحرشفية وسرطان خلايا الجلد القاعدية) وسرطان الميلانوما. تسبب خلايا الجلد منتجة في الأشعة فوق البنفسجية حوالي 90% من سرطان الجلد لان هذه الأشعة تسبب تلف دنا طفرات وراثية وتكوين سرطان الجلد.

المواد وطرق العمل: تتألف عينة الدراسة من 17 مريضا جمعوا بصورة عشوائية و 6عينات سيطرة لغرض المقارنة. أنجزت هذه الدراسة في مختبرات كلية العلوم / جامعة واسط بالتعاون مع مختبرات مستشفيات الكرامة والزهراء التعليميين في محافظة واسط للمدة من شهر تشرين الأول 2014 إلى نيسان 2015. تم دراسة تضاعف الجين *MDM2* بواسطة تقنية التهجين الموضعي الملون للدنا.

النتائج: أظهرت هذه الدراسة عدم وجود فرقا معنويا في تضاعف *MDM2* عند المقارنة بين عينة المرضى وعينة السيطرة($p>0.05$), بينما لوحظ وجود فرقا معنويا عاليا في كثافة تضاعف جين *MDM2* بين المرضى وعينة السيطرة ($p<0.05$). كذلك أظهرت النتائج وجود علاقة معنوية بين تضاعف الجين *MDM2* ونوع الأنسجة المصابة بسرطان الجلد . كما أظهرت الدراسة فروقا معنوية في تضاعف *MDM2*بين المرضى المصابون بسرطان الخلايا القاعدية و سرطان الخلايا الحرشفية و عينة السيطرة.

الاستنتاج: نستنتج بأن تضاعف جين *MDM2* كان عاليا في سرطان الخلايا القاعدية وسرطان الخلايا الحرشفية لان تضاعف *MDM2* في هذه الخلايا يقترن بمساهمتها في برنامج التمايز الخلوي.

الكلمات المفتاحية : *MDM2* , التهجين الموضعي الملون للدنا , سرطان الجلد

Abstract

Skin cancer: Skin cancer is the uncontrolled proliferation of abnormal skin cells; skin cancer is classified into non-melanoma (squamous cell carcinoma and basal cell carcinoma) and melanoma. Approximately, 90% of skin cancers are caused by ultraviolet radiation, because of ultraviolet radiation emits to damage of skin cells DNA resulting in genetic mutations and formation of skin cancer.

Materials and methods: This study evaluated paraffin blocks from 17 specimen randomly selected from patients with skin cancer, and 6 specimens from patients non cancer patients as control group for comparison .This study was carried out in Laboratories of College of Science, Wasit University and AL Zahraa and ALKarama Teaching Hospitals, from October 2014 to April 2015. In this study *MDM2*(murine double minute 2) gene amplification was assessed by chromogenic in situ hybridization (CISH) technique.

Results: This study showed that there was no significant difference between skin cancer patients and control group in relation to *MDM2* amplification ($p>0.05$), but presence of highly significant difference between patients of skin cancer and control group in relation to amplification intensity of *MDM2* ($p<0.05$). Moreover, and according to histological type, our finding found significant difference between the types of skin cancer in relation to *MDM2* amplification ($p<0.05$). Additionally, there were significant differences between patients of basal cell carcinoma and squamous cell carcinoma and control group in relation to *MDM2* amplification($p<0.05$).
Conclusion: We conclude that *MDM2* amplification was high in types of skin cancer (BCC and SCC) because of *MDM2* amplification in keratocytes of the epidermis may be associated with entry of these cells into the differentiation program.

Key word: Skin cancer, *MDM2*, chromogenic in situ hybridization.

Introduction

Skin cancer is the uncontrolled proliferation of abnormal skin cells: Skin cancer is classified into non-melanoma (squamous cell carcinoma and basal cell carcinoma) and melanoma (1). Approximately, 90% of skin cancers are caused by ultraviolet radiation (2), because of ultraviolet radiation leads to damage of skin cells DNA and formation the cyclobutane pyrimidine dimer (CPD) and the 6-4 photoproducts, causing genetic mutations resulting in atypical cellular proliferation and formation of skin cancer(3). Skin cancer represents one-third of all cancers that are diagnosed every year worldwide(4). Additionally, skin cancer constitutes nearly 40% of all diagnosed cancers (5). In Iraq (2009), skin cancer occupies the 8th position among the commonest ten cancers by primary site and gender, and that form about (3.99%), and in Wasit in same year, it occupies the 7th position among the commonest ten cancers by primary site and account (3.68%). Additionally, the highest incidence of skin cancer was in Duhok (12.3%), but the lowest incidence was in Basrah (2.41%) (6). On the other

hand, risk of skin cancer is increased in males more than females (7), because of males tend to have more exposure to sunlight than females (8). Additionally, people with darken skin tend to have a lower rates of skin cancer compared to peoples with white skin, because dark skin has larger number of melanocytes, which allows the dark skin to filter up ultraviolet B (UVB) radiation more than white skin people (9). Moreover, the incidence of skin cancer increases exponentially with age, because of accumulate exposure to UVR , and the declining ability to repair UV-damaged DNA are thought to contribute to an increased risk of skin cancer (10). Among of these genes which have role in this disease is *MDM2*. *MDM2* (murine double minute2) gene was found as an amplified sequence in a spontaneously transformed murine cell line, and shown to have tumorigenic potential. *MDM2* gene is located on chromosome 12q13-14(11). The major function of *MDM2* is inhibit the activity of the *p53* tumor suppressor (12). MDM2 protein plays a critical role in regulating cell proliferation and apoptosis (13). *MDM2* overexpression directly contributes to loss of p53 function during the development of nearly 50% of human cancers (14). This study aims to study the amplification of *MDM2* in skin cancer patients and control tissue sections, assessment the overlapping between the histopathological type of skin cancer and *MDM2* gene amplification and estimating the amplification intensity of this gene in patients and control group.

Materials and methods

Patients and tissue samples

Seventeen specimens of skin cancer's patients were taken randomly and six non cancer patients as control group for comparison. All specimens were collected from Al-Karama and Al-Zahraa Teaching Hospitals in Wasit Province, and from Medicine City in Baghdad. This study was carried out in Laboratories of College of Science, Wasit University and Al-Zahraa and Al-Karama Teaching Hospitals, from October 2014 to April 2015. Formalin fixed paraffin embedded tissue blocks were cut (4-5µm) thickness, from each tissue block. To simplify the statistical analysis, the patients were divided into histological types were taken in this study.

Procedure of the chromogenic in situ hybridization (CISH) technique to detect *MDM2*:

The kit which used in this work was provided by zytovision.

Serial tissue sections were cut 4-5µm thick and positioned on positive charged slides.

A-Dewax, Proteolysis (day1): The slides were baked in incubator at 65˚C over night. The tissue sections were deparaffinized; then the slides were rehydrated by graded ethanol concentration (100%, 90%, and 70%) and distal water. The slides were incubated in 3% Hydrogen peroxidase (H_2O_2) diluted in D.W. for 10 minute. After that, each slide was washed in D.W. twice for 1 minute. Place slides then in the pre-heated Heat Pretreatment Solution EDTA (**PT2**)

and incubated for l5 min. at 95°C. After that the slides were washed in 2x D.W., for 2 minute. The slides were incubated with 40µl of pepsin solution (**ES1**) for 5min. at room temperature in humidity chamber. The slides washed in 1x D.W., for 2 minute. Then dehydration of slides in 70%, 90% and l00% ethanol jars, each for1minute.

B- Denaturation and Hybri-dization (day1): Then the probe (*MDM2*) added on tissue section in slide and place cover slip on slide. The samples were covered with a cover slip. Then, seal the cover slip with a layer of hat glue from an adhesive pistol. Denature the slides at 78- 80°C for 5 min. on hot plate. After that, transfer the slides to a humidity chamber and hybridize overnight at 37°C.

C-Post-Hybridization and Detection (day2): Carefully remove glue, and cover slip by submerging in Wash Buffer SSC (**WB1**) at room temperature for 5 minute. Then the slides were washed for 5 min. in Wash Buffer SSC (**WB1**) at 75-80°C. The Wash Buffer SSC should-be pre-heated. Increase temperature by 1°C per slide for more than 2 slides. After that the slides were washed in 2x D.W., for 1min., and immerse slides then in1x Wash Buffer TBS (prepared using **WB5**) and drain off or blot off the 1 x Wash Buffer TBS,for 2minunte. Put 40µl of Anti-DIG/DNP-Mix (**ABI4**) to slides and incubated for 15 min. at 37°C in a humidity chamber. The slides were washed then in 2x1.5 min. in 1xWash Buffer TBS (prepared using **WB5**) and drain off or blot off the 1 x Wash Buffer TBS. Put 40 µl of HRP/AP-Polymer-Mix(**ABI3**) to slides and incubated for 15 min. at 37°C in a humidity chamber. After incubation the slides were washed in3x1min. in 1xWash Buffer TBS(prepared using **WB5**). Put 100 µl of AP-Red Solution to the slides and incubated for10 min. at RT (protect from strong direct light). After incubation the slides were washed in1x D.W. for 2 min. and drain off or blot off the water. Put 100 µl of HRP-Green Solution to the slides and incubated for10 min. at RT(protect from strong direct light). Then the slides were washed in1xD.W for 2 minunte. After that, counter stain the tissue or cell samples for 2 min with Nuclear blue solution (**CS2**).Transfer slides into a staining jar and washed 2 min in running tap water. Then dehydration of slides: 2x45 s. in 100% ethanol and 2x 30 s. in xylene (use very pure xylene). Cover the samples immediately with a cover slip by using Mounting Solution (alcoholic) (**MI4)** and air dry the slides for approx. 30 minute. Finally, evaluation of the slides were carried out by light microscopy. In normal diploid nuclei without chromosomal aberrations, 2 green and 2 red distinct dot-shaped signals with smooth, rounded edges will be visible per nucleus.While, with chromosomal aberrations, a different signal pattern can be visible (15). Green dot due to using HRP-Green solution, and Red dot due to using AP-Red solution.

Ethical consent

The study was submitted and approved by the College of Science, University of Wasit in collaboration with AL-Karama and AL- Zahraa Teaching Hospitals, Wasit - Iraq.

Statistical analysis

For all statistical analysis, the SPSS system for personal computer was used, and p values of 0.05 or less were regarded as statistically significant. Sensitivity and specificity of the tests (with 95% exact confidence intervals) were determined in studied group. Comparison between groups was carried out using Chi-square test. Correlation and Fisher's exact test, Binary Logistics Regression analysis were also performed whenever appropriate.

Results

Chromogenic in situ hybridization (CISH) analysis of *MDM2* amplification was reported in 14 (82.4%) of skin cancer patients out of 17cases (fig1), and 3(50%) of control group out of 6 cases. There was no significant difference between skin cancer patients and control group in relation to *MDM2* amplification ($p>0.05$). Intensity assessment of *MDM2* amplification in patients showed that 3(17.6%) cases were non-amplification, 8(47.1%) cases were high level of amplification and 6 (35.3%) cases were low level of amplification. While, assessment of *MDM2* amplification intensity in control group were 3(50%) out of 6 cases were non-amplification, 0(0%) cases were high level of amplification and 3(50%) out of 6 cases were low level of amplification. There was a highly significant difference between patients of skin cancer and control group in relation to amplification intensity of *MDM2* ($p<0.05$) (Table 1). According to histological type, *MDM2* amplification was reported in 9(90%) of basal cell carcinoma out of 10 cases, and in 4(100%) of squamous cell carcinoma out of 4 cases. While our results showed amplification of *MDM2* 1 (33.3%) of malignant melanoma out of 3 cases. There was significant difference between the types of skin cancer in relation to *MDM2* amplification ($p<0.05$) (Table2).

Amplification of *MDM2* in patient with basal cell carcinoma, showed in 9(90%) out of 10 cases, while in control group, amplification of *MDM2* was in 3 (50%) out of 6 cases. There was significant difference between BCC patients and control group in relation to *MDM2* amplification ($p<0.05$) (Table3).

Moreover, *MDM2* amplification in patient of squamous cell carcinoma was in all cases 4(100%) , while in control group, amplification of *MDM2* was in 3(50%) out of 6 cases .There was highly significant difference between SCC patients and control group in relation to *MDM2* amplification ($p<0.05$) (Table4).

Table (1):*MDM2* amplification and intensity in skin cancer patients and control group:

Samples	*MDM2* amplification			*MDM2* intensity			Total No %
	No%			No%			
	Non-amplification	Amplification	Total	Non	High level	Low level	
Patients	3 (17.6%)	14 (82.4%)	17 (100%)	3 (17.6%)	8 (47.1%)	6 (35.3%)	17 (100%)
Control	3 (50%)	3 (50%)	6 (100%)	3 (50%)	0 (0%)	3 (50%)	6 (100%)
Total	6	17	23 (100%)	6	8	9	23 (100%)
P>0.05				P<0.05			
(non significant)				(P=0.001,highly significant)			

Table(2): *MDM2* amplification in skin cancer patients according to histological type:

Type	Non-amplification No%	Amplification No (%)	Total No (%)
BCC	1(10%)	9(90%)	10(100%)
SCC	0(0%)	4(100%)	4(100%)
MM	2(66.7%)	1(33.3%)	3(100%)
Total	3	14	17(100%)
P<0.05			

(significant)

Table(3): *MDM2* amplification between BCC patients and control group:

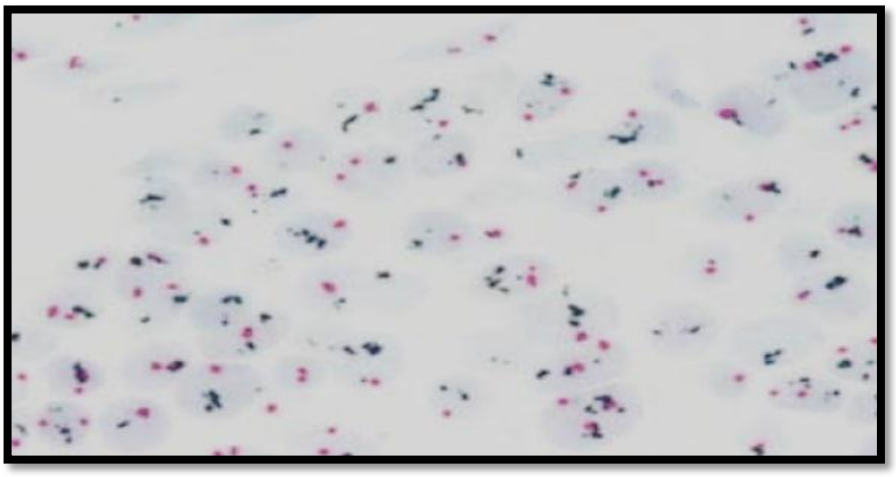
Sample	Non-amplification No (%)	Amplification No (%)	Total No(%)
BCC	1(10%)	9(90%)	10(100%)
Control	3(50%)	3(50%)	6(100%)
Total	4	12	16(100%)
P<0.05			

(significant)

Table(4): *MDM2* amplification between SCC patients and control group:

Sample	Non-amplification No (%)	Amplification No (%)	Total No(%)
SCC	0(0%)	4(100%)	4(100%)
Control	3(50%)	3(50%)	6(100%)
Total	3	7	10(100%)
P<0.05			

(p=0.007 , highly significant)



Figure(1): Chromogenic in situ hybridization examination showing positive amplified *MDM2* status in skin cancer (x100).

Discussion

In this study, the difference in amplification of *MDM2* between patients and control group cases is no significant, but there was highly significant difference in *MDM2* intensity between both groups. Moreover, result of Ganguli *et al*.(12) showed that *MDM2* was expressed highly in the suprabasal layer and less in the basal layer of the epidermis of human skin, by using immunohistochemistry technique. This due to *MDM2* expression in keratocytes of the epidermis may be associated with entry of these cells into the differentiation program (16).

Also, our results showed that amplification of *MDM2* was strong in BCC and SCC compared to MM. Moreover, results of Rajabi *et al*.(17) explained that the rate of expression of *MDM2* by using of immunohistochemistry technique was low in malignant melanoma 12 (28%) out of 43 cases, and they suggested that the *MDM2* is not an appropriate diagnostic factor in MM. On the other hand, low rate of *MDM2* expression was associate with tumor thickness and invasion level.

Overall ,*MDM2* express highly in MM, because of apoptotic function inhibition of p53 in MM through induction of *MDM2*,which expressed in melanoma (18).

Additionally, the present result showed significant difference between BCC patients and control group in *MDM2* amplification. Ganguli *et al*.(12) showed that *MDM2* was expressed highly in normal human skin in the suprabasal layer and less in the basal layer of the epidermis , by using immunohistochemistry technique. While, no other results were obtained to compare between SCC patients and control group in *MDM2* amplification, but this is may be lead to the fact that the *MDM2* is highly amplified in epidermis layers which is contained the squamous cells and basal cells.

As a conclusion, the present study showed that *MDM2* amplification was high in types of skin cancer (BCC and SCC) because of *MDM2* amplification in keratocytes of the epidermis may be associated with entry of these cells into the differentiation program.

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