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Histopathological and molecular characterization of Egyptian's non melanoma skin cancer

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ABSTRACT: In humans, the most prevalent malignant neoplasms are non-melanoma skin cancers (NMSC). Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) account for approximately 95% of all NMSC skin malignancies. In Egypt, sun exposure is responsible for more than 60% of NMSC, while skin color accounts for about 45%. The present study aimed to find sharp and accurate molecular technology to diagnose NMSC beside histopathology. 20 patients of NMSC patients were randomly selected. Tissues skin biopsies were collected from tumor and free areas for histopathological investigation and molecular analysis by random amplified polymorphic DNA (RAPD). Histopathological results revealed that 55.0% of NMSC patients had SCC, 45.0% had BCC. 35.0% of the patients were in early stage and 65.0% were in late stage, but 15.0% were in low grade and 75.0% were in high grade. 90.0% of the cases weren't metastasized by the tumor while 10.0% were invaded by the tumor. RAPD analysis by OPA-02 primer found a significant (P=0.007) tumor marker band (2000 bp) for SCC patients and no significant bands for BCC. The study succeeded in introducing RAPD for detection of skin cancer beside histopathology.

Keywords: Non melanoma skin cancer; Skin cancer histopathology; RAPD analysis of skin cancer; Skin basal cell carcinoma; Skin squamous cell carcinoma.

1. INTRODUCTION

Cancer of the skin is wide spread concern to the health care system that is expected to increase dramatically over the following two decades if it is not detected early [1]. The etiology is mostly based on aberrant growth of skin cell promoted by unresolved DNA in the cells of skin due to mutations of DNA or genomic instabilities. Skin tissue consists of 2 layers; the upper layer called epidermis, which is built up of pigmented melanocytes and epithelial cells and the lower layer called dermis, which is a of connective tissue layer that is rich with sweat glands, hair follicles and blood vessels [2-4].

Non-melanoma skin cancer (NMSC), which arises from the epidermis, is the type of cancer of the skin that is most commonly found globally. Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) are the main subtypes of NMSC. People with multiple myeloma had a 26.8% increased risk of acquiring skin cancer than those without multiple myeloma (16.1%). NMSC seldom travels down into the innermost layers of the epidermis if ignored, and it can only be efficiently eradicated when discovered at an early stage [5]. BCC is the most common kind of skin cancer in humans, with around 4.3 million cases per year. The greatest risk is exposure to sunlight. The rate of death is poor but the illness rate is significant as a result of tissue destruction

[6]. Approximately 50% of all skin cancer patients have BCC. BCCs are substantially more common in Caucasians [7-9]. Overexposure to UV rays is the primary cause of SCC, which typically affects light-skinned individuals. Other factors that contribute to SCC include aging, burn scars, chronic skin ulcers, immune suppression, chemical carcinogens, and a variety of molecular pathways [10]. Malignant melanoma is cancer cells that form as the consequences of aberrations in skin melanocytes. Melanoma is an aggressive malignant tumor and accounts for 75% of all deaths [11].

BCC located in the bottom layer of epidermal cells are also keratinocytes. BCC is mostly caused by long-term sun exposure, insufficient immunity to cancer-inducing factors, beta human papilloma virus (HPV) and human immunodeficiency virus (HIV) [12]. When organisms are exposed to ultraviolet radiation, an accumulation of genetic changes occurs, activating oncogenes, deactivating tumor suppressor genes, and inhibiting DNA repair. This pathway may lead to unregulated melanocyte proliferation and eventually cancer [13]. Chromosome aberrations, insertions, amplifications, translocations, mutations, and deletions are all examples of genetic changes. These mutations can occur as a result of exposure to various external and internal stressors, and they can be corrected by DNA repair systems or accumulate in cells [14]. UV-specific mutations in the p53 tumor suppressor gene, found in 50% of BCCs, are another common mutation [15].

According to Egypt's national cancer registry, which is based on data from 40,477 patients collected retrospectively from Tanta cancer center over a 9-year period, skin cancer incidence was calculated as 3.1% of all cancers in both sexes and 3.8% of all cancers in males based on the population of Gharbia (as an indicator of rates in Egypt) and the number of cases of each cancer type there [16]. Sun exposure was responsible for more than 60% of NMSC in the Egyptian population, while skin color accounting for around 45% [17]. The National Population-Based Cancer Registry Program (2008-2011) identified NMSC as accounting for 1.35% of all malignancies in males and 1.24% in females [18]. Early detection of skin cancer is important in treating the problem very fast by complete body skin inspection, dermoscopy, and biopsy histopathology [19].

With the rising prevalence of skin diseases and skin cancer, skin biopsy is becoming a more common medical procedure. Histopathological examination of skin cancer biopsy determines the tumor type, grade and stage. Tumour staging is based on the American Joint Committee on Cancer (AJCC) classification which additionally considers tumour thickness, perineural infiltration diameter, and tumour invasion depth to classify primary tumours. The presence of metastases following a selective sentinel node biopsy was predicted by the Brigham and Women's Hospital (BWH) and AJCC systems [20].

DNA fingerprinting is emerging to be of great significance in the establishment of the paternity of an individual. Human genetic variation at the DNA level is explained firstly by Professor Sir Alec. He discovered the mini satellite region, which was isolated and used as a probe to investigate human DNA. It was found that the mini satellite probe was a complex pattern of bands for each individual, and was detecting a large number of hyper variable regions in the human DNA. These regions were interrelated, and each band represented one of the hyper variable DNA regions. Further refinement and sequencing led to recognition of unique identification pattern for every individual namely a DNA Fingerprint. DNA fingerprinting has been included into the standard work of challenged paternity cases as a powerful technique of investigation in forensic cases, notably in the field of forensic medicine and genetics [21].

One of several DNA fingerprinting PCR (polymerase chain reaction) based methods is RAPD analysis. The RAPD marker application methodology uses arbitrary oligonucleotide short primers, typically 8 to 15 nucleotides long, to randomly amplify DNA regions of massively genomic DNA by PCR. Prior DNA sequence data is not required in RAPD analysis due to the use of random primers. RAPD fragments can be easily isolated

in agarose gels using electrophoresis [22]. Studying genetic alteration of breast cancer is studied by RAPD [23]. Also, ovarian cancer [24], bladder tumors [25], renal tumors [26] and the genomic variability of *H. pylori* [27] are studied by the same technique. Genetic polymorphisms and alterations in human tumors such as lymphoma, SCC of the head and neck, leukemia, brain tumor, hepatocellular carcinoma and lung cancer are examined by RAPD analysis [28].

2. MATERIALS AND METHODS

2.1. Patients

The study was a pilot case-control study and included 20 patients with ages ranging from 40-80 years. The study comprised 11 male and 9 females, of them 11 diagnosed as squamous cell carcinoma and 9 as basal cell carcinoma. The patients were randomly selected from the oncology center, Mansoura University, Egypt.

2.2. Study protocol

Studied patients were classified into two groups. The 1st group was 11 patients (7 males and 4 females) of different grades and stages of SCC and their ages were ranging from 40-80 years. The 2nd group included 9 patients (5 females and 4 males) of different grades and stages of BCC and their ages were ranging from 40-82 years.

2.3. Samples collection

Skin cancer patients were hospitalized into the oncology center, faculty of medicine, Mansoura University, Egypt. During skin tumor resection surgery, normal and tumor tissue specimens were collected freshly from each patient. Each specimen is divided equally. The first half was homogenized by tissue homogenizer (Mechanika Precyzyjna, Poland) for RAPD fingerprinting analysis, while the second part was stored in 10% formalin (Sigma-Aldrich, USA) for histopathological processing.

2.4. Histopathological characterization

Histopathological steps for slide preparation was started with tissue processing in tissue processor (Sakura, Japan), embedding the tissue samples in paraffin blocks by blocks forming apparatus (Tissue -Tek, Japan), cutting the paraffin blocks into 3-5 µm thin sections by microtome (Reichert Jung 2030, West Germany) by using disposable blade (Reichert, low profile, West Germany). Hematoxylin and eosin stains (BDH, U.K) preparation, staining methods and slides mounting were accomplished according to approved protocol [29].

2.5. Molecular analysis

2.5.1. DNA extraction and quantization

DNA was extracted from homogenized tissues by phenol-chloroform isoamyl alcohol manual method [30]. Modified phenol-chloroform extraction method was used in extracting the genomic DNAs from patient's tumor and normal tissue (negative control). The extracted genomic DNA concentration and purity was assessed by nano drop spectrophotometer (Thermo Scientific 2000, USA). The optimum purity ratio was in-between 1.8-2.0. The extraction of purified, high quality DNA is a prerequisite for performing genetic fingerprinting studies. Extracted genomic DNA of both tumor and normal samples were electrophoresed in 0.7% agarose.

2.5.2. DNA electrophoresis

Extracted pure genomic DNA integrity was assembled through electrophoresis in 0.7% agarose (Roko, Spain). In microwave, 0.35 gm agarose was cooked in 50 ml of 1x Tris Acetate EDTA (TAE) (Sigma-Aldrich, USA), let to cool to 60°C and mixed with 5 µl Midori green advanced DNA stain (Nippon, Germany) and poured into gel casting tray containing comb. The gel was left to polymerize and each DNA samples (3 µl) were mixed with 1 µl bromophenol blue (6x) loading dye (Sigma-Aldrich, Germany) and loaded into the formed gel wells after removing the comb. 3 µl Lambda DNA/Hind III ladder marker (Sigma-Aldrich, Germany) was loaded into the first well of the gel. Electrophoresis was done in gel electrophoresis apparatus (Labnet, UK) for 45 min at 100 V in 1x TAE, the electrophoretic mobility of the DNA was determined against the DNA marker and demonstrated on ultraviolet light box (Fotodyne, USA).

2.5.3. RAPD-PCR with kit-A primers

The primer that put to use as a diagnostic primer should reveal a high degree of genomic diversity that can be occurred by producing a large number of loci during the amplification process. Five different arbitrary of decamer oligonucleotides of kit-A (Eurofins Genomic, Germany) primers (A-01 to A-05) were screened.

2.5.3.1. Reaction mixture preparation

DNA amplification was carried out with five primers of kit-A; A-01 (5'-CAGGCCCTTC-3'), A-02 (5'-TGCCGAGCTG-3'), A-03 (5'-AGTCAGCCAC-3'), A-04 (5'-AATCGGGCTG-3'), A-05 (5'-AGGGGTCTTG-3') to examine the primers kit efficiency. PCR mixture was prepared in 200 µl sterile PCR Eppendorf by mixing 1 µg of pure genomic DNA, 2 units of Taq DNA polymerase enzyme (Viviantis, Malesia), 30 pm of each primer, one PCR bead (Enzynomics, Korea), sterile deionized water was added to reach 20 µl final volume and 20 µl mineral oil (Sigma- Aldrich, Germany) was dropped over the PCR mixture.

2.5.3.2. RAPD-PCR cycling condition

PCR Eppendorf containing the components was then transferred into the PCR machine (ProFlex™, Singapore) which was set into three programs. 1st program was cycled for 40 cycles; each cycle was programmed into three steps; 95 °C for 2 min, 30 °C for 1 min and 72 °C for 2 min. The 1st program was linked to the 2nd program (72 °C for 7 minutes) for the final DNA extension. The 2nd program was linked to 3rd program (hold at 4 C°). The result of RAPD-PCR was analyzed through electrophoresis in agarose gel a described up with some modifications; agarose gel was prepared as 2% and the DNA marker (GeneDirex, USA) was 1kb DNA ladder. DNA bands molecular weights was calculated by GellApp software 1.2.7 Android app.

2.6. Statistical analysis

GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, California, USA) was used for all statistical analyses. Results were presented as the mean ± the standard error of the mean (SEM) (n = 6). One-way analysis of variance (ANOVA) was used to do statistical comparisons, followed by the Neuman-Keule post-hoc test [31]. When the P value was < 0.05, a significant difference was considered, and any higher significance level was indicated. Chi-squared analysis and Fisher's exact test (lower than 20 cases) were used to analyze the relationship between genomic instability, histological type, and grade of skin cancer. A significant correlation, $P < 0.05$ was considered acceptable. GellApp software 1.2.7 android app was used to determine the molecular weight of the amplified DNA fragments, including tumor and normal DNA bands through blotting a curve illustrating the detected fragment molecular weights with respect to the marker fragments molecular weight.

3. RESULTS

3.1. Patients data

Patient's data including; number, age, sex, tumor type and the sex were recorded in Table 1.

Table 1. Sex distribution of NMSC patients.

Characteristic	Statistics
Number	20
Age (mean ± SD, range/ years)	68 ± 10 (40- 85)
Sex (male/female)	11/9
SCC	
Number	11 (55%)
Sex (male/female)	7 /4 (63.6% / 36.4%)
BCC	
Number	9 (45%)
Sex (male/female)	4/5 (45.4% / 54.6%)

3.2. Histopathology results

Histopathology investigations including the tumor site, size, grade, stage and if there are any lymph nodes were metastasized with the tumor were demonstrated in Table 2 and histopathology features of skin tissue were demonstrated in Figure 1.

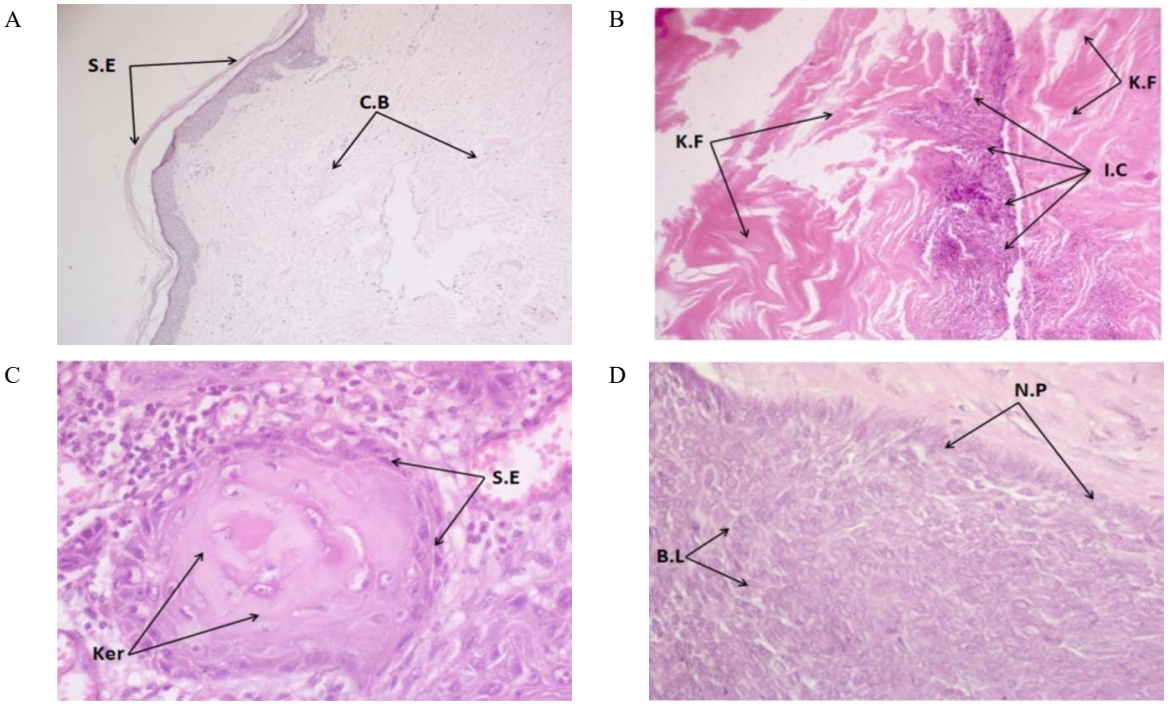


Figure 1. Histopathology feature of skin tissue stained with hematoxylin and eosin stain, x100 magnification; A - Normal skin tissue lined with mature squamous epithelium (S.E) overlying connective tissue with collagen bundles (C.B.); B - Inflamed skin tissue showing keratin filaments (K.F) with infiltrating inflammatory cells (I.C); C - Well differentiated squamous cell carcinoma tissue with easily recognizable squamous epithelium (S.E), abundant keratinization (Ker), intercellular bridges apparent and minimal pleomorphism; D - Basal cell carcinoma tissue with large basaloid lobules (B.L) and peripheral nuclear palisade (N.P). Pleomorphism is generally mild with variable mitotic activity and apoptosis.

Table 2. NMCS histopathology.

Parameter	Number (Percentage)
Tumor size	
Small (≤ 3 cm)	13 (65.0%)
Large (> 3 cm)	7 (35.0%)
Tumor site	
Head and Neck	13 (65.0%)
Others	7 (35.0%)
Tumor stage	
Early (T ≤ 2)	7 (35%)
Late (T > 2)	13 (65%)
Tumor grade	
Low (G1)	5 (25%)
High (G2-G3)	15 (75%)
Lymph node invasion	
Negative	18 (90.0%)
Positive	2 (10.0%)

3.3. Molecular results

3.3.1. RAPD- PCR analysis of SCC and BCC

Genomic DNAs were isolated from normal and tumor tissues of the different groups (BCC and SCC) and amplified using OPA-02 primer to give different RAPD fingerprints bands. 7 bands (8135, 4057, 2900, 2000, 1580, 1350 and 1015 bp) were appeared in SCC, while 6 bands (5000, 2700, 1800, 1340, 1137 and 844 bp) were appeared in BCC. RAPD-PCR analysis of SCC elevated only one significant (P = 0.007) tumor marker band (2000 bp) in 90.9% of the tumor samples and disappeared in 64.0% of normal samples, while six insignificant bands were appeared (Figure 2, Table 3). On the other side, RAPD-PCR of BCC by A-02 primer appeared six insignificant bands, can't produce any differences between normal and tumor tissues of the same patients. (Figure 3, Table 4).

Table 3. Number, percentages, P value and Chi-square of amplified DNA fragments that generated by A-02 primer of SCC tumor and normal tissues.

SCC (no= 11)				
DNA bands molecular weight/ (bp)	**Tumor samples No and (%)**	**Normal samples No and (%)**	**X^2**	**P**
8135	1 (9.0)	1 (9.0)	0	1.0
4057	0 and 0	1 (9.0)	0	1.0
2900	10 (90.9)	10 (90.9)	0	1.0
2000	10 (90.9)	4 (36.0)	7.0714	0.007*
1580	3 (30.0)	0 and 0	2.3294	0.12695
1350	4 (36.0)	0 and 0	3.6667	0.055511
1015	3 (30.0)	0 and 0	1.2222	0.268925

* Significant P value = P < 0.05.

Table 4. Number, percentages, P value and Chi-square of amplified DNA fragments that generated by A-02 primer of BCC tumor and normal tissues.

BCC no = 9				
DNA bands molecular weight/ (bp)	**Tumor samples No and (%)**	**Normal samples No and (%)**	X^2	**P**
5000	1 (11)	0 and 0	0.4	0.527089
2700	5 (55.0)	6 (66.0)	0.23	0.627
1800	6 (66.0)	7 (77.0)	0.276	0.598
1340	2 (22.0)	0 and 0	1.0	0.317
1137	1 (11)	1 (11)	0.0	1.0
844	1 (11)	0 and 0	0.4	0.527089

Significant P value = $P < 0.05$.

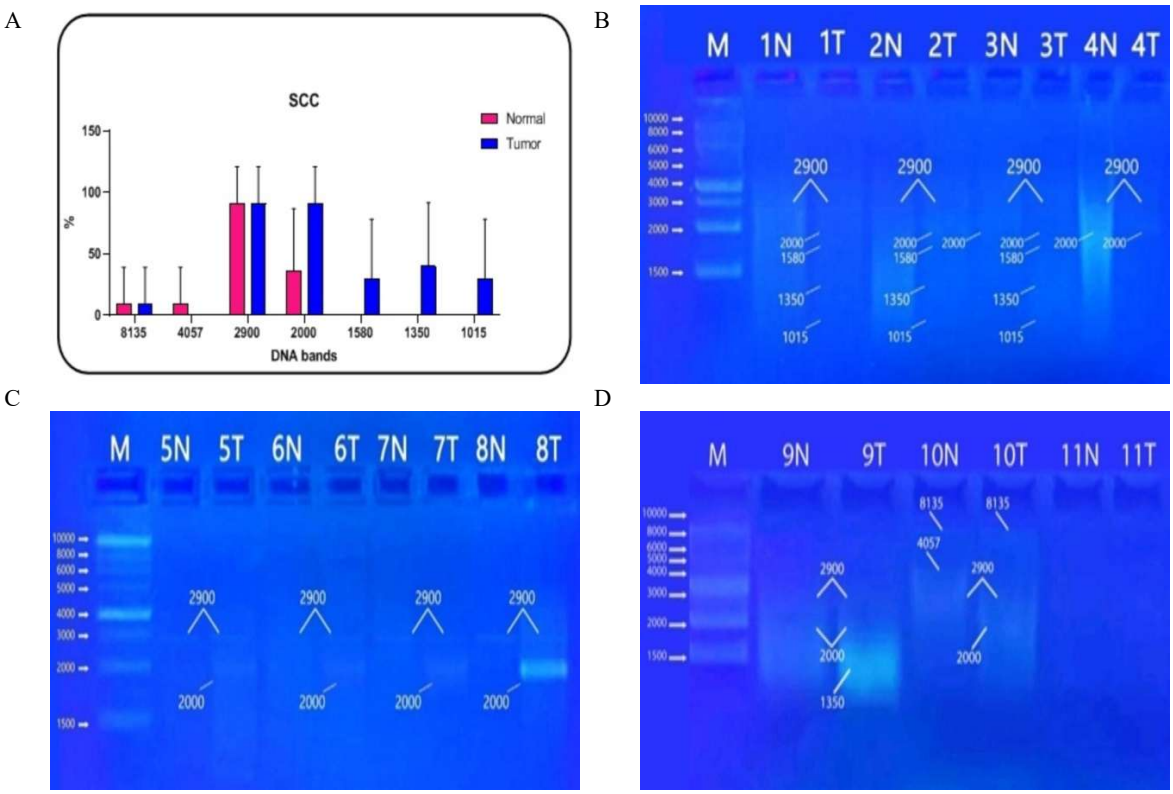


Figure 2. Molecular characterization and agarose gel electrophoresis of SCC RAPD-PCR product of samples amplified by primer A-02, at 100 volts in 2% agarose and 1x TAE buffer for 2 hrs. M: 1Kb DNA ladder marker (250-10000) bp; A - Bands molecular weight percentages calculated by Gel App analyzer; B - samples 1-4; C - samples 5-8; D - samples 9-11.

4. DISCUSSION

Skin cancer is considered as one of many different sorts of cancer that can exist due to uncontrolled skin tissue growth. 18.1 million people are given a skin cancer diagnosis each year [32]. The current study is going in parallel with a previous study [18] in which NMSC occurred in roughly equal levels in both sexes. This might be related to the way women's life styles are evolving in Egypt, where more women are working outside, particularly in agriculture.

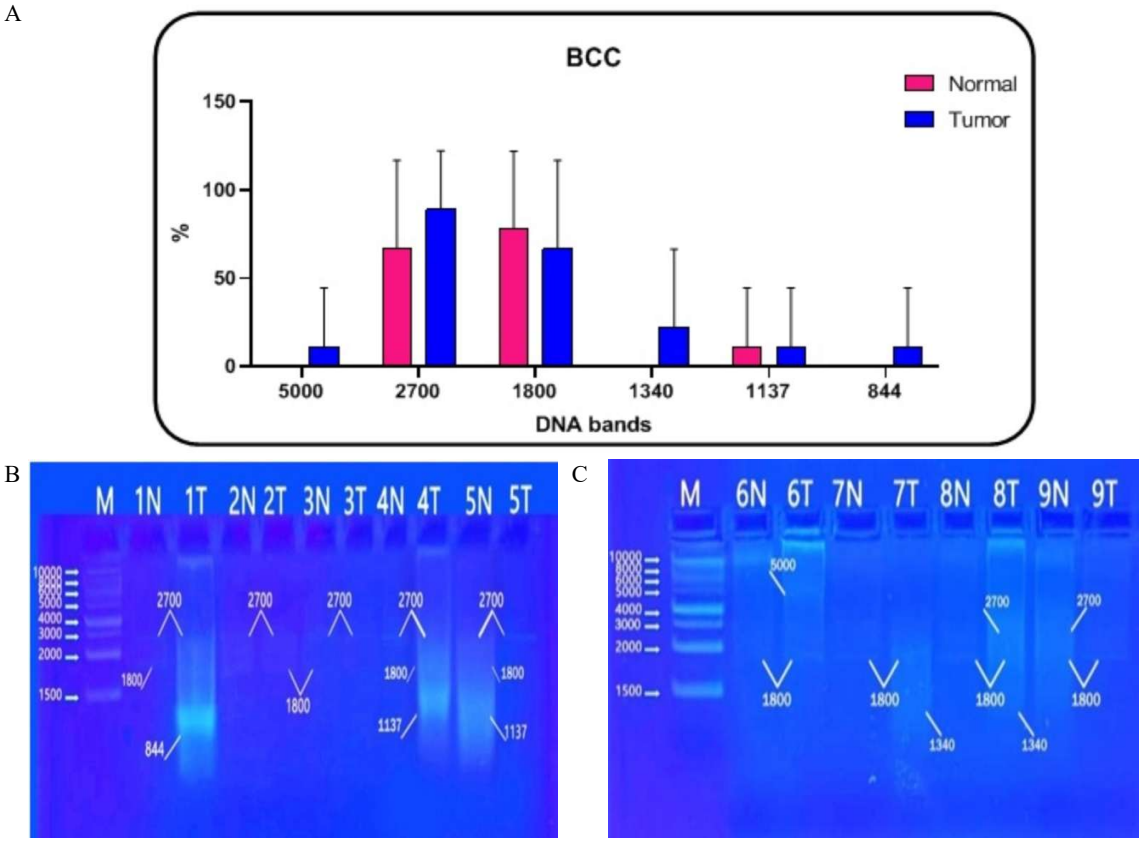


Figure. 3. Molecular characterization and agarose gel electrophoresis of BCC RAPD-PCR product of samples amplified by primer A-02, at 100 volts in 2% agarose and 1x TAE buffer for 2 hrs. M: 1Kb DNA ladder marker (250-10000) bp; A - DNA bands molecular weight percentages calculated by Gel App analyzer; B - samples 1-5; C - samples 6-9.

Another study [17] illustrated that; approximately 60% of NMSC cases in Egyptians may be related to exposure to sunlight and nearly 45% to skin colorization. These previous study results are matching with our study results in which the majority of NMSC were discovered on skins that overexposed to sunlight, mostly on the neck and head (65%) specially in farmers and employees in welding industries. Additionally, we found that the patients' age range average was about 68 ± 10 years and about 70.0% of the NMSC patients were above 50 year matching with a former study [33].

National comprehensive cancer network recommended 4-6 mm for relatively safe tumors; 10 mm for extremely dangerous tumors. Tumor location, size, characteristics, invasion of neighboring tissues and histological classification are used to classify high- and low-risk groups [34]. But the present study revealed that the skin tumor size ranges between 65% and 35% for minor (the size ≤ 3 cm) and large (size > 3 cm) tumors, respectively. Furthermore, depth of invasion was recorded for each tumor as tumor stage. This was calculated as the how far it is from the deepest portion of an ulcer to the deepest portion of the tumor [35]. The current study expressed that the measured average depth of invasion at early stage (T ≤ 2mm) were approximately 35% and in late stage (T > 2mm) were 65%.

We performed RAPD-PCR analysis of genomic DNA obtained from twenty NMSC patients tumor tissues and their adjacent normal tissue was performed by using 10-base pair random primer (A-02) with GC rich sequence (5'-TGCCGAGCTG-3'). Five insignificant (P > 0.05) bands 8135, 4057, 2900, 1580, 1350 and 1015 bps bands were appeared in squamous cell carcinoma in normal and tumor tissues samples in frequent

percentages. On the other hand, a significant (P = 0.007) tumor band with molecular size 2000 bps was appeared and could be utilized as a diagnostic genetic marker band for squamous cell carcinomas skin cancer patients. Six insignificant (P > 0.05) bands 5000, 2700, 1800, 1340, 1137 and 844 bps were appeared in BCC normal and tumor samples in different percentages. SO, A-02 primer failed to produce any significant genetic differences between normal and tumor of BCC skin cancer patients. Another study [36] used the same primer A-02 (5'-TGCCGAGCTG-3') that we used to amplify the extracted DNAs from squamous and basal cell carcinomas as well as cutaneous malignant melanoma and two similar bands 1018 and 1636 bp were appeared. The study supported our results in BCC, in which the median amount of tumor DNA changes demonstrated a noticeable gain of bands.

5. CONCLUSION

RAPD-PCR approach generated specific bands associated with skin cancer. Appearing of new skin cancer band/s or disappearing of normal skin band/s is a marker for genetic instability and cancer development. The present study used RAPD fingerprinting analysis which revealed a promising molecular technology for skin cancer diagnosing beside the histopathology which considers the gold stander for cancer diagnosis. The study is preliminary and further molecular studies are needed for demonstration and identification of skin cancer.

Conflict of Interest: The authors of this work declare that they have no conflicts of interest.

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Ethics statement: Skin cancer patients were hospitalized into the Oncology Center, Faculty of Medicine, Mansoura University, Egypt. Informed consent was obtained from all participants and/or their legal guardians. Skin tumor resection surgeries were routine clinical procedures; therefore, approval from the bioethics committee was not required.

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