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Original Article

Histopathological investigation of Skin Cancer and Study of *CDKN2A* Gene Polymorphism in Mazandaran Province, Iran

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Skin cancer is one of the most important type of cancers in the world. In this way, molecular investigation in order to detect some novel mechanisms and polymorphisms involved in cancer development can be impressive and vital. In this way, the aim of this study was the histopathological investigation of skin cancer and its relationship with polymorphism of *CDKN2A* gene. This case-control study was conducted in north of Iran and 36 samples of squamous cell carcinoma (SCC), 64 cases of basal cells carcinoma (BCC) and 50 healthy control samples were investigated. Histopathological and tissue structures were evaluated after hematoxylin and eosin staining, under light microscope. In addition, investigating the *CDKN2A* gene polymorphism was carried out by using PCR_sequencing. 58% of patients were male. The mean age of patients was 63.69 years and the average tumor size was 3.57 mm. No *CDKN2A* gene polymorphism was observed in intron 1. The investigation of other DNA repair genes can help to better understanding of genetic factors predisposing to skin cancer development.

Keywords: Histopathology, polymorphism, *CDKN2A* gene, basal cell carcinoma, squamous cell carcinoma

Skin cancer is one of the most common kinds of cancers in most countries with genetic and environmental factors playing an important role in its development (1-2).

Given that these cancers are classified into several types, the molecular and histopathological mechanisms of the genes are different. So that, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) as non-melanoma skin cancer (NMSC) and malignant melanoma skin cancer (MSC) have different prevalence figures (3). Statistics indicate that the incidence of this cancer in different regions are different and also in this classification the BCC and SCC types represent 80% and 20% of cases,

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respectively (4). In contrast, melanoma is a rare type of skin cancer but it is one of the deadliest forms of cancer (5).

In general, there are different molecular pathways and a large number of genes including *CDKN2A* involved in the development of skin cancer and especially melanoma (6). Molecular studies have presented evidence of the relationship between family melanoma and *CDKN2A* involvement which is a tumor suppressing gene located on the chromosome 9p21 (7-8). This kinase coding gene is dependent on P16 cyclin inhibitor that inhibits kinase-dependent cyclin 4 and 6 (CDK4 & CDK6) and as a result it activates the retinoblastoma protein family that controls G1 to S phase transfer in the cell cycle. Lack of P16 performance can lead to uncontrolled cell multiplication and eventually cause neoplasm. *CDKN2A* / *P16* somatic mutation has been documented in a large variety of human tumors (9-18).

Given the importance of this type of cancer, molecular genetics research can be very useful in determining the involved cancer genes and identifying new polymorphisms. The aim of this study was to evaluate the tissue of skin cancer histopathologically and identify new polymorphisms of the *CDKN2A* gene in this type of cancer.

Materials and methods

Sampling

In this study 100 paraffin-embedded skin cancer samples and 50 control samples from subjects referred to the Bou-Ali Sina hospital, Sari, Mazandaran, Iran, for skin diseases evaluation during 2005 to 2015, were collected from the archives of the department of pathology. In this way, samples were biopsied based on gender, age, skin cancer type, tumor size and the location of the tumor. Patients were classified into 5 age groups, including: 20-40, 41-50, 51-60, 61-70 and 71-90 years old. Also due to the dispersion of the tumor location, their positioning was classified into 4 groups: head and neck, hands, feet and body. The cancer tissues included 100 non-melanoma tissues that have been divided into 64 BCC and 36 SCC.

Histopathological investigation

The samples were cut by microtome (Leitz 1512, Germany) into 3-6 µm in diameter. Hematoxylin and eosin (H&E) staining was performed. After staining, the slides were analyzed in terms of histopathological features by 10 X and 40 X optical microscopy magnifications.

DNA Extraction

Samples were deparaffinized by xylene 100% (Merck, Germany). Then the tissue was disrupted by lysis buffer and proteinase K and the DNA was extracted using the DNA extraction kit (Takapoozist, Iran). Then, to ensure the quality of the extracted DNA, they were electrophoresed on 1% agarose gel. Correspondingly, for quantity analysis, their OD was measured by a spectrophotometer (Eppendorf Biophotometer, Germany) at 260/280 nm wavelength.

Amplification and sequencing of *CDKN2A* gene

CDKN2A gene sequence was obtained from GenBank (NCBI) and rs36168473 located in the first intron (accession number NC_000009.12: g.21980971delT) was studied after sequencing. Oligo V7.56 software was used for designing the specific primers. Remarkably, a 643 bp fragment was amplified using forward 5' TGATTACTGT AGGCTCCACTC 3' and reverse 5' GTCAAG AGCATGTATTGGTAAC 3' primers (Bioneer, Korea) in a final volume of 25 µl after an initial

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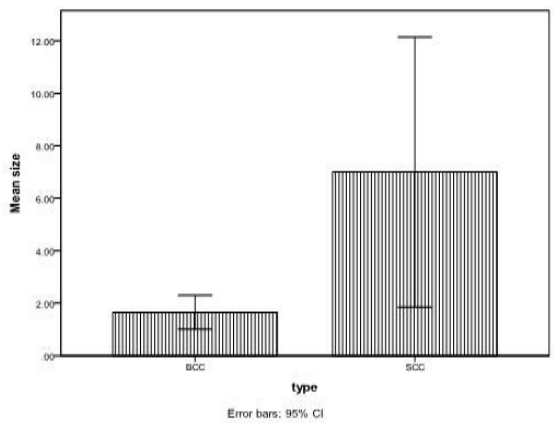


Figure 1. Size of biopsied tumors in BCC and SCC types. Average tumor size in BCC was less than 2 mm and about 7 mm in SCC.

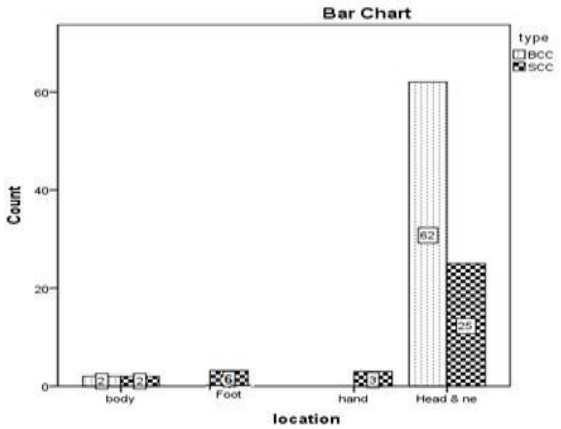


Figure 2. Tumor location frequencies. Tumors were located into four regions including body, feet, hands, head and neck.

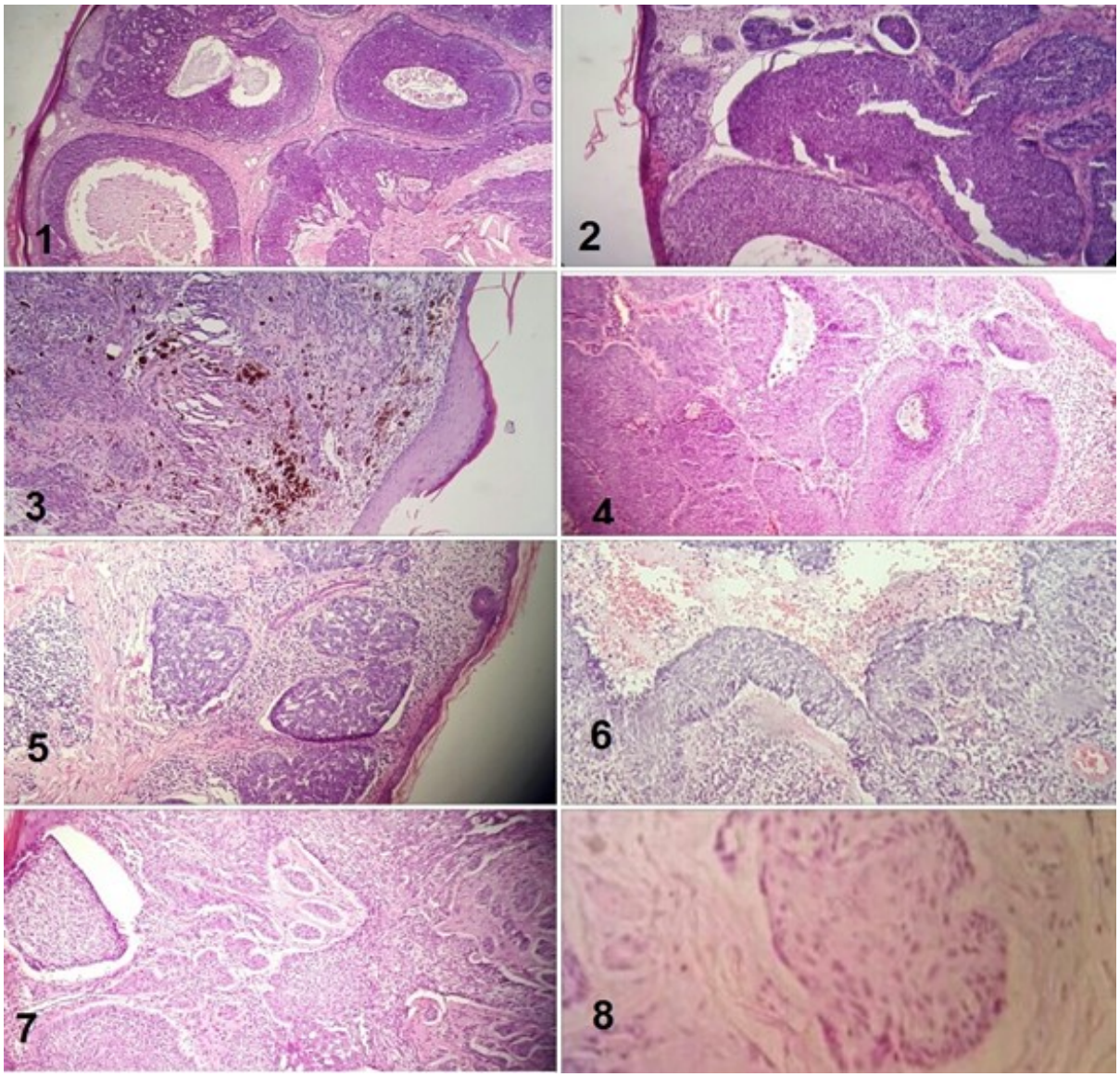


Figure 3. BCC skin cancer samples. 1: cystic basal layer tumor; 2: cystic tumor cells, gaps around the tumor and palisade peripheral cells; 3: tumor cells in the form of nest and cork along with melanin pigment production; 4: basaloid tumor cells and their association with epidermis; 5: basal tumor cells' infiltration and its association with the epidermis; 6, 7: tumor cells' infiltration and their association with the epidermis; 8: a tumor cell nest with the gap around the nest being a palisade. (10 X magnification)

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denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min.

The quality of PCR products was analyzed by electrophoresis on 1% agarose gel. PCR products were sequenced using the forward primer (Bioneer Company, Korea). Sequencing results were analyzed by the CLC Main Workbench bioinformatics software.

Results

Histopathological findings

The study was conducted on 100 patients with skin cancer and 50 normal subjects as controls. The patients included 58 men (58%) and 42 women (42%). The mean age of patients was 63.69 years. Patients were classified into 5 groups, including: 20-40 (n=4, 4%), 41-50 (n=10, 10%), 51-60 (n=32, 32%), 61-70 (n=21, 21%) and 71-90 (n=33, 33%) years old. Most patients were within the age range of 51 to 60 and 71 to 90 years.

The nodular tumor type had the highest frequency in BCC while the well differentiated type

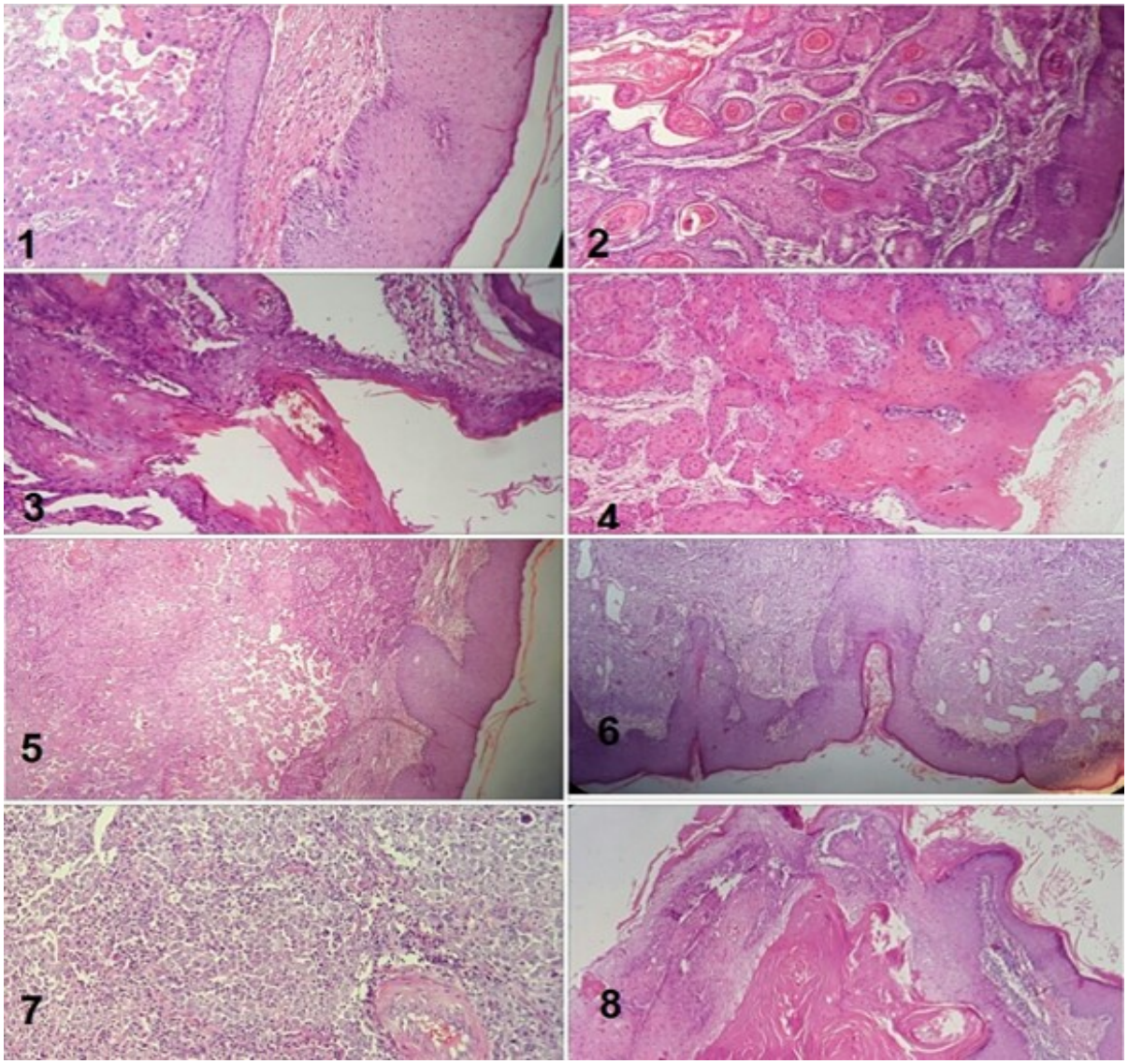


Figure 4. SCC skin cancer samples. 1: dermis tumor cells connected to epidermis; 2: keratin production in tumor cells; 3: cells and cell plates in the tumor; 4: tumor cells as the nest and cell plates with keratin; 5, 6, 8: dermis tumor cells connected to the epidermis; 7: pleomorphism and hyperchromasia of the nuclei. (10 X magnification)

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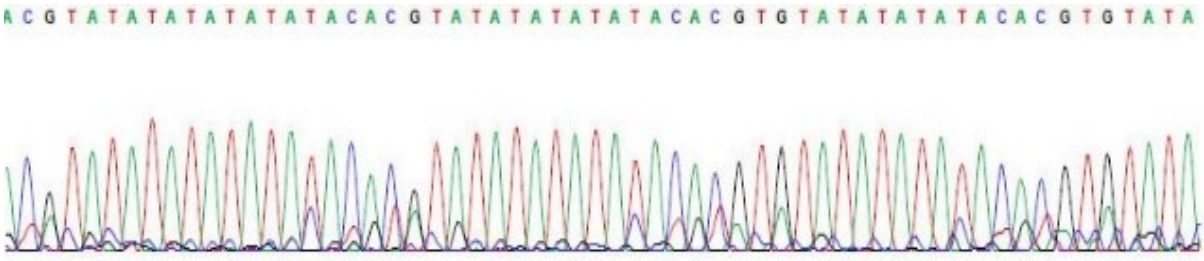


Figure 5. A representative sequencing electrophoregram. No deletion was observed at rs36168473.

had the highest frequency in SCC. The size of the biopsied tumor was on average 1.652 mm in BCC and 7.294 mm in SCC (Figure 1). Tumors were present in the body, feet, hands, and head and neck regions. The frequency of BCC and SCC tumors in the head and neck was 62% and 25%, respectively (Figure 2).

Histologically, BCC tumor cells formed the dermal nests, cords and islands that were associated with the epidermal region in some cases. Cells that made up the nests were small, round and similar to basal keratinocytes. Around nests, cells were dispersed in parallel directions such that they formed figures like a palisade (Figure 3-2 and 3-8). Cells had low pleomorphism and mitosis. Accumulation of melanin pigment in some clinical brown lesions was obvious (Figure 3-3). The cystic space in the tumor nests which might be due to the necrosis at the center or frills was also observed (Figure 3-1 and 3-4).

SCC tumor cells also formed the dermal nests, cords and islands that were associated histologically with the epidermal region. Tumor cells were large and multi-dimensional and had cell-cell junctions. Keratin production can be seen in tumors which have a better differentiation (Figure 4-2 and 4-4), and also nuclei had different degrees of pleomorphism and mitosis (Figure 4-7). Accumulation of melanin pigment in some lesions caused clinically brown color.

Molecular analysis of rs36168473 at *CDKN2A* locus

In order to study rs36168473 polymorphism the samples were sequenced (Figure 5). No polymorphism was observed for rs36168473 at intron 1 of *CDKN2A* gene in all analyzed samples.

Discussion

According to the findings of the present study which was conducted between 2007 to 2015 in the north of Iran, non-melanoma skin cancer (NMSC) was seen in 100% of the cases among which BCC was more prevalent than SCC. The mean age of patients was 63.69 years. The BCC and SCC types were observed in 64% and 36% of patients, respectively. Average tumor diameter was about 3.54 mm. Tumors were located in head and neck (87%), body (4%), feet (6%) and hands (3%). Therefore, most tumors were observed in head and neck areas that are more exposed to the sun due to the climatic conditions in this region. Skin cancer usually appears at high age and the average age of the disease is 50 and 60 years and one of the important environmental factors inducing these cancers is the ultraviolet light. In another histopathological analysis conducted in Turkey among 136 patients, 127 were diagnosed with NMSC. The mean age of the patients was 68.5%. The BCC and SCC type were observed in 75.6% and 24.4% of patients, respectively. The mean tumor size was between 3.49 and 7.42 mm, respectively in BCC and SCC types. Tumors were observed in the face (67.6%), scalp (11.8%) and ears (11%) (19).

The molecular investigation of polymorphism

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in intron 1 of *CDKN2A* gene in non-melanoma cancer patients as well as normal subjects in the present study revealed no polymorphism at rs36168473.Yaman in a comparative study analyzed the somatic mutations in genes such as *CDKN2A* in primary cutaneous melanoma and found that after *BRAF* (42.5%) and *NARS* (15.1%) gene mutations, *CDKN2A* gene mutation with 13.2% prevalence was the most common mutation (20). In another population-based study performed on 759 DNA repair genes in relation to NMSC, an intronic single nucleotide polymorphism (SNP) rs4135150 was highly unbalanced (21). However, in *CDKN2A* gene, polymorphism in intron area (rs36168473) was not observed. Pjanova analyzed *CDKN2A* gene mutations in patients with melanoma as well as other cancers in which the whole gene coding region was displayed by polymorphism analysis and DNA sequencing and found no genetic mutations in patients with melanoma (22).

Finally, the comparison of the frequency of skin cancer lesions and different genetic polymorphisms indicates that the genetic and environmental factors have a significant impact on the frequency of this type of cancer in different regions.

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Conflict of interest

The authors declared no conflict of interest.

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