Original Paper

P21 Immunoexpression in Actinic Keratosis and Cutaneous Carcinomas

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ABSTRACT: Actinic keratosis is a precancerous lesion of the skin with variable rates of transformation into non-melanocytic carcinomas. The present study aims to analyze p21 expression in actinic keratosis and cutaneous squamous carcinomas. P21 expression analysis revealed a positive reaction in 63 (71.4%) of the investigated cases. The immunostaining analysis revealed positivity in 82.1% of the studied actinic keratosis cases and in 43.9% of the investigated carcinomas. High scores of immunoreactions were identified only for the well-differentiated and for the carcinomas in initial stages. Association of p21 expression with pre-invasive or well-differentiated invasive lesions in early stages of tumor progression suggests that p21 may be an early event in squamous carcinogenesis.

KEYWORDS: *actinic keratosis, cutaneous squamous carcinomas, p21 immunoexpression*

Introduction

Actinic keratosis is a precancerous lesion of the skin that develops after long-term sun exposure [1]. So far, different series have reported variable rates of transformation into non-melanocytic cancers of the skin. The progression rate to squamous cell carcinoma varies between 12 and 20% [2,3]. Some studies have estimated the risk at 0.075-0.06% per lesion per year or about 1% within 10 years [4] with an estimate of up to 10% over 10 years [5]. Even though only a small number of actinic keratosis seem to develop into an invasive squamous cell carcinoma, patients have multiple lesions and often from many years and thus have a much greater risk of developing squamous cell carcinoma.

The abnormal cell cycle control is closely related to carcinogenesis. The p21 protein, produced by the TP21/WAF1 (wild-type activating fragment) gene, is a member of the tumor suppressor gene family, which acts as a CDK (cyclin-dependent kinase) inhibitor and is essential for cell growth, differentiation and apoptosis. Expression of p21 is regulated by p53 in response to DNA (deoxyribonucleic acid) damage. Then, p21 associates with CDK, preventing the phosphorylation of its substrates and blocking the cell cycle progression. This arrest gives the cell time to repair DNA, thus preventing replication of damaged genetic material [6].

The present study aims to analyze p21 expression in actinic keratosis and cutaneous squamous carcinomas with varying degree of differentiation and tumor stages.

Material and methods

The study comprised 89 cases, of which 28 actinic keratosis cases with varying degrees of severity and 61 cases of squamous carcinomas with varying degrees of differentiation. The analyzed cases were obtained from the Clinics of Dermatology and Surgery of the Emergency Clinical Hospital Craiova. The surgical incision specimens were fixed in 10% buffered formol, then processed by the classic histopathological technique and hematoxylin-eosin stained. The lesions' classification was made according to the literature recommendations [7,8] (Table 1).

Table 1. Distribution of the analyzed cases depending of the lesion type

Lesion	Actinic Keratosis			Squamous cell carcinomas		
	KIN I	KIN II	KIN III	WD	MD	PD
No. of cases	22	3	3	18	37	6

*KIN-keratinocyte intraepithelial neoplasia; WD-well differentiated; MD-moderate differentiated; PD-poorly differentiated

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The investigated squamous carcinomas corresponded in 18 cases to well-differentiated forms (16 in Stage I and 2 in Stage II), in 37 cases to moderately differentiated (32 in Stage I, 4 in Stage II and 1 in Stage III), and in 6 cases to poorly differentiated tumors (3 in stage I, 2 in stage II and 1 in stage III).

Seriated sections were subsequently processed for immunohistochemistry using a polymer amplification based detection system (Histofine Horseradish Peroxidase conjugated polymer, Nichirei, Japan, ready to use, code 414151F). For visualization of the reactions, DAB (3,3'-Diaminobenzidine) chromogen (code 3467, Dako) was used, and positive (colon adenocarcinoma) and negative (by omitting the primary antibody) external controls were used to validate the reactions. In this study we use a monoclonal mouse anti-human anti-p21 antibody (Biocare Medical), clone WA-1, diluted as 1:50, with microwaving in citrate buffer pH 6 as antigen retrieval.

We followed the semi-quantitative p21 expression through an adapted scoring system that was independently assigned by two specialists (CS and AS) based on the staining intensity and the percentage of positive cells [9]. The intensity score was considered 1 (low intensity), 2 (moderate intensity) and 3 (strong intensity). The cut off value for the positivity of the reactions was set at 5%. The percentage of colored cells was scored as 1 (6-25% positive cells), 2 (26-50% positive cells), 3 (51-75% positive cells), and 4 (>75% positive cells). The multiplication of the intensity and percentage scores allowed the obtaining of the final staining scores (FSS), which was considered low for values between 1-4 and high for values between 6-12. The statistical analysis used average values and comparison tests (ANOVA, chi square-χ 2) in the SPSS10 automated software. The study was approved by the local ethical committee (no.171/11.09.2017), and a written informed consent was obtained from all the patients.

Results

P21 expression analysis revealed a positive reaction with variable scores in 63 (71.4%) of the investigated cases. The staining distribution was observed both nuclear and cytoplasmic

The analysis of the p21 positive cells percentage and the intensity of positivity revealed the presence of the marker for the 23 cases of actinic keratosis respectively 82.1% of the studied cases. The marker distribution was observed in basal keratinocytes, isolated or in small groups, as well as in rare cells in the upper layers of the epidermis, heterogeneous (Fig. 1A). Also, the staining were observed in the stromal elements. The p21 final staining score (FSS) had values between 1 and 2 with an average of 1.1 for KIN I and 1.3 for KIN II and respectively KIN III (Table 2). Statistical analysis did not reveal statistical associations of KIN grade with p21 expression (p=0.231, Anova test).

Table 2. Distribution of the actinic keratosis cases depending of p21 FSS

DEGREE OF KIN	KIN I	KIN II	KIN III
FSS / no. of positive cases	1,1/17	1,3/3	1.3/3

*FSS-final staining scores; KIN-keratinocyte intraepithelial neoplasia

For the investigated cases of squamous carcinomas, we noticed the positive reaction in 40 tumors, representing 65.5% of the investigated carcinomas cases. We noticed quite high variations in the FSS values of p21 depending on tumor grade and stage (Table 3).

Table 3. Distribution of the squamous carcinomas cases depending on p21 FSS

DEGREE / STAGE	WD	MD	PD
Stage I: FSS / no. of positive cases	4.4/13	2.3/19	2.5/2
Stage II: FSS / no. of positive cases	3/1	2/2	1/1
Stage III: FSS / no. of positive cases	0/0	4/1	1/1

*WD-well differentiated; MD-moderate differentiated; PD-poorly differentiated; FSS-final staining scores; KIN-keratinocyte intraepithelial neoplasia

Thus, regardless of the differentiation degree and stage, we noticed the p21 positivity for these tumors. The immunostainig were particularly intense for differentiated tumor cells, which showed keratinization and less often in undifferentiated epithelial tumoral cells. In all analyzed cases the p21 signals were also identified in the stromal elements, the skin annexes and the adjacent or suprajacent epidermis of the tumors.

In well differentiated squamous carcinoma cases, the p21 staining had a heterogeneous pattern and variable intensity (Fig. 1B). For moderately and poorly differentiated forms of squamous carcinomas, the p21 staining identified the presence of reaction both in the peripheral as well as a random pattern in neoplastic cell islets with low or moderate intensity (Fig. 1C-D).

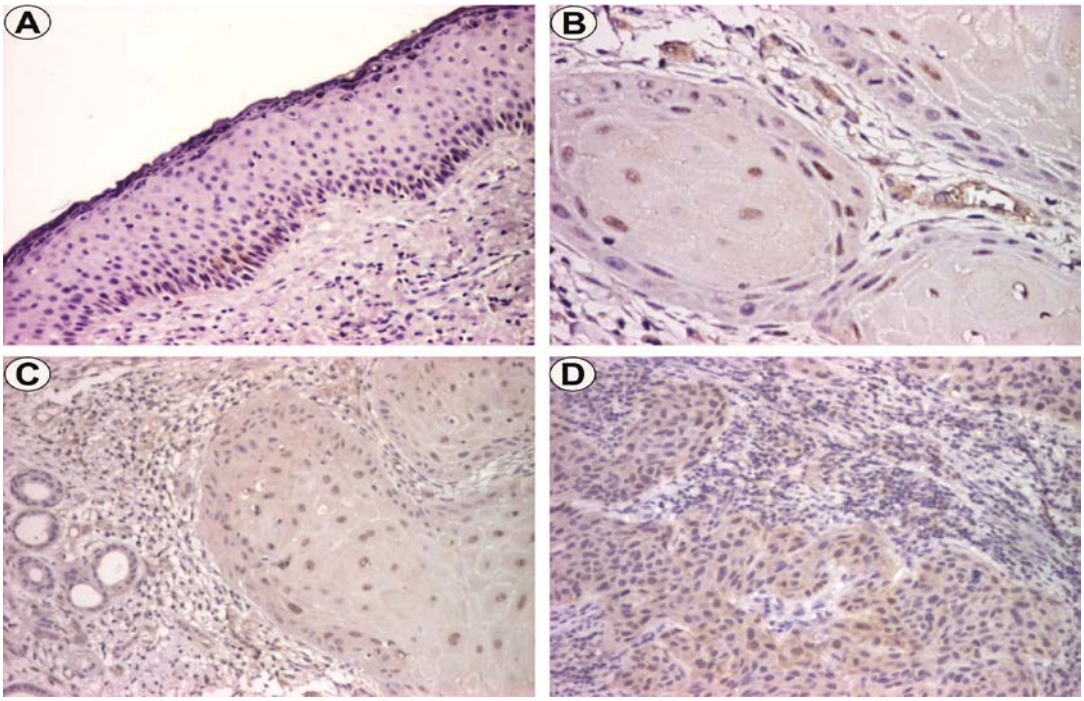

Fig.1. p21 immunostaining:
A. Actinic keratosis (KIN I), x100; B. well differentiated squamous carcinoma, x100;
C. moderate differentiated squamous carcinoma, x100; D. poorly differentiated squamous carcinoma, x100

The FSS values for the investigated squamous carcinomas ranged from 1 to 9. For stage I tumors, FSS values were 4.4, 2.3, and 2.5, respectively in well, moderately and poorly differentiated forms. For the stage II tumors, FSS values were 3, 2, and 1, respectively, in well, moderately and poorly differentiated forms, and in stage III tumors, the FSS was 4 in moderately differentiated carcinomas and 1 in poorly differentiated carcinomas.

The statistical analysis indicated significant differences in p21 expression related to carcinoma differentiation (p=0.000, χ 2test), p21 high scores being observed only in case of well differentiated carcinomas (Fig. 2A). Although p21 high scores were only observed in early stages, we did not find any statistical correlation between p21 and the tumor stage (p=0.536, χ 2 test) (Fig. 2B)

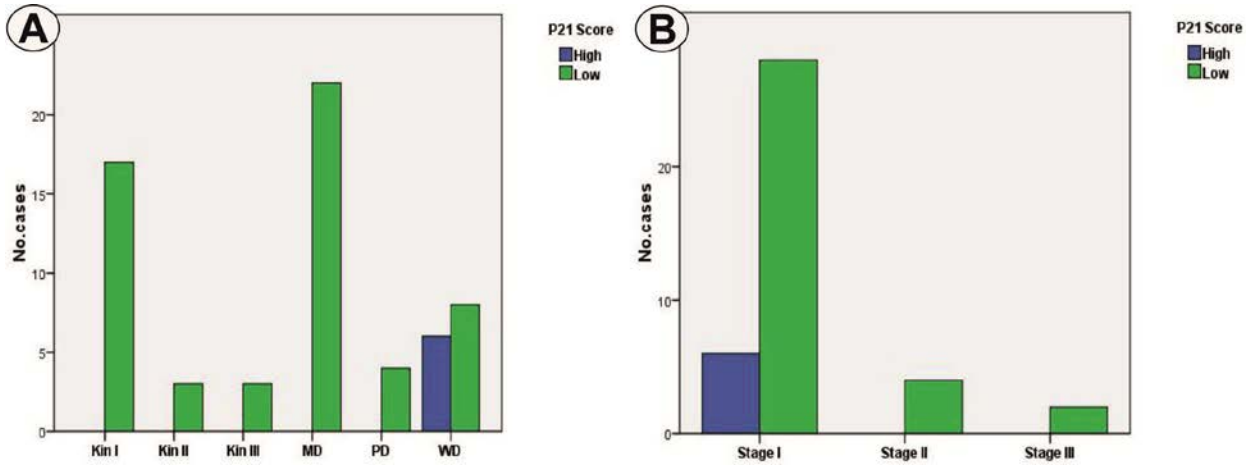

Fig.2. Distribution of the positive p21 carcinomas depending on differentiation degree (A) and tumor stage (B)

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Discussions

The p21WAF1/CIP1 (wild-type activating fragment-1/cyclin-dependent kinase inhibitory protein1) protein is a universal inhibitor of cyclin-dependent G1 kinase and is induced by p53-dependent or independent pathways. The p21 protein exists in a quaternary cyclin complex, a cyclin-dependent protein kinase (CDK) and the cellular proliferation nuclear antigen (PCNA). It has been suggested that p21 mediates the shut-down of p53-induced cell growth triggered by DNA damage [10]. The p21 protein has been shown to stop p53 activation due to decreased cell proliferation [11]. Also, p21 has been reported to decrease cell proliferation and activate p53 blocking [11,12].

In normal skin, p21 expression was negative in the epidermis, but present [13] in sweat glands, sebaceous glands, capillary endothelium and smooth muscles [14] more intense compared to skin neoplasia. A small number of studies reported the role of p21 in the dedifferentiation of cutaneous squamous carcinomas [15].

For this study, p21 expression analysis indicated positivity for this marker in 23 of the actinic keratosis investigated cases. The marker has been identified isolated in keratinocytes or in small groups of squamous cells, as well as in rare cells in the upper layers of the epidermis, heterogeneous. In the literature, other studies report similar aspects, in positive actinic keratosis, observed only in basal and suprabasal epidermal layers, as well as in the upper spinocellular layer [16]. Some studies reported progressive positivity for p21 in the keratin layer in relation to the cell maturation, and in cases of normal skin, hyperplasic or border lesions, in well and moderate differentiated squamous carcinomas a higher expression, probably due to the high percentage of the differentiated squamous component [17].

For squamous carcinomas, we observed positivity in 40 of the carcinoma cases, majority in Stage I and II tumors, regardless of degree of differentiation. Statistically significant high scores of p21 immunoexpression have been identified in well differentiated carcinomas compared with high grade lesions. Also, the scores were superior in initial stages compared with advanced ones, but without statistical significance. In one study, the authors found heterogeneous positivity for p21 for half of the squamous carcinoma, regardless of the differentiation degree [12]. Such aspect is quite unexpected because p21 is a known inhibitor of CDK activity, which stimulates the progression of the cell cycle. As a result, p21 could be an early event in squamous carcinogenesis without being directly associated with the progression of these malignant tumors [18]. This is supported by the frequent intense staining of the p21 protein in precancerous lesions [12,19]. Similar findings are reported in the study by Brasanac et al. who reported immunostaining for p21 Cip1/Waf1, more frequently in actinic keratosis than in squamous carcinoma in situ ($p=0.001$) and invasive lesions ($p=0.0004$) [20].

Conclusions

The association of p21 expression with actinic keratosis and well differentiated squamous carcinomas from the early stages of tumor progression suggests that p21 could be an early event carcinogenesis, without being directly associated with the progression of squamous cell carcinomas.

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