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Original article

Metastasis suppressor proteins in cutaneous squamous cell carcinoma

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ABSTRACT

Cutaneous squamous cell carcinomas (cSCCs) are common human carcinomas. Despite having metastasizing capacities, they usually show less aggressive progression compared to squamous cell carcinoma (SCC) of other organs. Metastasis suppressor proteins (MSPs) are a group of proteins that control and slow-down the metastatic process. In this study, we established the importance of seven well-defined MSPs including NDRG1, NM23-H1, RhoGDI2, E-cadherin, CD82/KAI1, MKK4, and AKAP12 in cSCCs.

Protein expression levels of the selected MSPs were detected in 32 cSCCs, 6 in situ SCCs, and two skin cell lines (HaCaT, A-431) by immunohistochemistry. The results were evaluated semi-quantitatively using the HSCORE system. In addition, mRNA expression levels were detected by qRT-PCR in the cell lines.

The HSCOREs of NM23-H1 were similar in cSCCs and normal skin tissues, while RGHOGDI2, E-cadherin and AKAP12 were significantly downregulated in cSCCs compared to normal skin. The levels of MKK4, NDRG1 and CD82 were partially conserved in cSCCs. In stage I SCCs, nuclear staining of NM23-H1 (NM23-H1nuc) was significantly lower than in stage II/III SCCs. Only nuclear staining of MKK4 (MKK4nuc) showed significantly higher scores in in situ carcinomas compared to invasive SCCs.

In conclusion, similar to other human tumors, we have demonstrated complex differential expression patterns for the MSPs in in-situ and invasive cSCCs. This complex MSP signature warrants further biological and experimental pathway research.

1. Introduction

Non-melanoma skin cancers (NMSC) are the most common human malignant neoplasms, and create significant medical, economical, and social problems for the healthcare services worldwide [1–3]. Although there are other types of NMSC, this term commonly refers to the two common neoplasms of cutaneous squamous cell carcinoma (cSCC) and basal cell carcinoma (BCC) [1]. BCCs are slow growing, malignant, yet rarely metastasizing carcinomas [4]. On the contrary, cSCCs are more aggressive in behavior, and have considerably higher metastatic capacities than BCCs [4].

Metastasis is a very complex and multistep biological process directed by various proteins and pathways [5]. Though various proteins support metastasis, a group of proteins called metastasis suppressor proteins (MSPs) specifically inhibit or slow metastasis [6,7]. As a definition, pure MSPs should only suppress metastasis without any effect on tumorigenicity (e.g. proliferation). However, in the complex environment of a cell, they usually have other important properties as tumor suppressor activities, affecting carcinogenesis, besides these MSP functions [6]. cSCCs differ from internal identical organ cancers in that they have lower metastatic rates and result in better prognosis [4]. Thus, cSCCs are interesting biological models for the research of metastasis suppressors. To establish the importance of the MSPs in non-melanoma skin cancer, we selected seven essential and well-defined MSPs (NDRG1, NM23-H1, RhoGDI2, E-cadherin, CD82/KAI1, MKK4, and AKAP12) that affect different steps of metastasis.

The main aim of this study was to analyze the expression patterns of these seven important MSPs that may contribute to the inhibition of metastasis pathways in cSCCs, as well as in squamous cell lines. We also established the association between

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these proteins and important clinicopathological parameters in cSCC.

2. Materials and methods

2.1. Study groups

A total of 38 SCCs composed of 32 tissue samples of classical squamous cell carcinoma (SCC-NOS) of the skin and 6 in situ carcinoma tissues, obtained from 37 patients (26M/11F), were included in this study. All patients were Caucasians, and the detailed characteristics of the study group are summarized in Appendix 1. SCCs were graded by four-tiered system as well (Grade 1), moderately (Grade 2) poorly differentiated (Grade 3) and anaplastic or undifferentiated tumors (Grade 4) [8]. Data for the SCCs were collected by using the CAP (The College of American Pathologists) protocols for squamous carcinoma of the skin (www.cap.org).

2.2. Normal skin control group

Ten normal skin tissues (4M/6F) from reconstructive operations, confirmed as normal by microscopy, were included as the normal tissue group. The normal group included skin tissues from different localizations; two from the face, three from the extremities, three from the breast, and two from the abdomen. This control group was the same as the one used in another previous study of our group [9]. Normal, non-lesional epidermis adjacent to SCC (N-SCC) was also integrated in this study.

2.3. Cell lines

The normal immortalized keratinocyte cell line (HaCaT) and the vulvar squamous carcinoma cell line (A-431) were used for immunohistochemistry and quantitative reverse transcriptase-PCR (qRT-PCR) studies [10,11].

2.4. Immunohistochemistry technique and analysis

The immunostaining procedure was performed by the classical labeled streptavidin-biotin immuno-enzymatic antigen detection system (UltraVision-Thermo Scientific; Waltham, MA, USA) with DAB chromogen, in the Thermo-Shandon Sequenza® manual staining station (Waltham, MA, USA). The primary antibody step was skipped for the negative control. The sources of primary antibodies and the technical details are demonstrated in Appendix 2.

All immunohistochemically stained slides were evaluated by external and internal controls. Stained slides were semi-quantitatively evaluated using a specific immunohistochemical histological score technique, (HSCORE), with minor modifications. The analysis technique was described in detail in the literature and in our previous study [9,12].

2.5. Cell culture

The A-431 cell line was cultured in Dulbecco's Minimal Essential Medium (DMEM-low glucose, Hyclone-Thermo Scientific, Waltham, MA, USA), and HaCaT cell line was grown in DMEM-High glucose (Hyclone), supplemented with 10% fetal bovine serum (FBS) and 50 mg/ml penicillin/streptomycin, at 37 °C, and in 5% carbon dioxide.

2.6. Real-time PCR (qRT-PCR)

500 ng of total RNA was reverse-transcribed using oligo-dT primers with the First Strand cDNA Synthesis Kit (Fermentas, Thermo, Waltham, MA, USA). All qRT-PCR experiments were performed using the SYBR® Green reagent (Thermo) in an MX3005P thermocycler (Strategene®, Agilent, Santa Clara, CA, USA). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *HPRT1* (hypoxanthine phosphoribosyl transferase 1) reference genes were selected for normalization. Data was analyzed using the free REST© 2009 software (Qiagen, Hilden, Germany) [13]. The sequences of primers used are accesible from our previously published paper [9].

2.7. Statistical analysis

Statistical analyses were performed using the PASW® Statistics 18 software (Chicago, IL, USA). The differences between the HSCOREs of the groups were first analyzed with the non-parametric Kruskal–Wallis one-way analysis test; followed by Mann–Whitney *U* test was applied. $p \leq 0.05$ was accepted as significant. The "Bonferroni correction" was used to reduce type I errors. The correlation between the parameters was analyzed by Spearman's correlation test, where $r \geq 0.25$ and $p \leq 0.05$ were accepted as significant.

2.8. Ethics statement

This study was financially supported by the Scientific and Technical Research Council of Turkey (TUBITAK, grant number SBAG-108S184). The project was approved by the Kırıkkale University Local Ethics Committee (07.04.2008/2008-039).

3. Results

3.1. Immunohistochemical staining results

3.1.1. Tissue study

Cytoplasmic positivity of NM23-H1 (NM23-H1$_{cyt}$) and NDRG1 (NDRG1$_{cyt}$) was detected to be strong and homogeneous in all of the in situ and invasive cSCCs (Fig. 1A, B, F, G). However, nuclear NM23-H1 (NM23-H1$_{nuc}$) expression was significantly weaker and was detected in only two of in situ SCCs (IS-SCC, 33.3%) and 15 cSCCs (46.8%) compared to normal skin. Nuclear positivity of NDRG1 (NDRG1$_{nuc}$) was seen in 4 of 6 IS-SCCs (66.6%), and 29 of 32 cSCCs (90.6%).

E-cadherin, CD82 and AKAP12 proteins show only weak and medium cytoplasmic-membranous staining (Figs. 2A, B, F, G and 3A, B). E-cadherin and CD82 positivity was detected in all of IS-SCCs, but AKAP12 positivity was observed in 4 IS-SCCs (66.6%). E-cadherin, CD82, and AKAP12 expressions were detected in 84.3%, 68.7% and 40.6% of the cSCCs, respectively.

RHOGDI2 showed cytoplasmic positivity in 5 (83.3%) and 30 (93.7%) of the in situ and invasive cases, respectively, though with weak to medium strength only (Fig. 3F, G). Nuclear positivity of RHOGDI2 (RHOGDI2$_{nuc}$) was weaker, yet more heterogeneous, in 4 of 6 IS-SCCs (66.6%) and 14 of 32 cSCCs (43.7%).

MKK4$_{cyt}$ was detected in all of IS-SCCs, and weak MKK4$_{nuc}$ was detected in 5 of 6 IS-SCCs (83.3%) (Fig. 4A). MKK4 immunostaining of cSCCs showed weak to medium cytoplasmic positivity in 25 cases (78.1%), and weak nuclear positivity in 7 cSCCs (21.8%) (Fig. 4B).

3.1.2. Cell lines

NM23-H1 showed strong cytoplasmic positivity in the both HaCaT and A-431 cell lines (Fig. 1C, D). However, focal nuclear positivity was stronger in the HaCaT cell line. Significant positivity was observed with NDRG1 antibody in both of the cell lines (Fig. 1H, I). E-cadherin expression was stronger in the HaCaT cell line compared to A-431 (Fig. 2C, D). CD82/KAI1 showed medium levels of positivity in both cell lines (Fig. 2H, I). RHOGDI2 showed strong but heterogeneous positivity in the HaCaT and A-431 cell lines (Fig. 3H,