Pathology – Research and Practice 210 (2014) 596–602

Contents lists available at ScienceDirect

Pathology – Research and Practice

journal homepage: www.elsevier.com/locate/prp

Original Article

Pattern of TGFbeta receptor 1 expression differs between *kras*-mutated keratoacanthomas and squamous cell carcinomas of the skin

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ARTICLE INFO

Article history:
Received 19 September 2013
Received in revised form 26 April 2014
Accepted 15 May 2014

Keywords:
Skin carcinogenesis
TGFbeta
EGFR
Keratoacanthoma
Squamous cell carcinoma

ABSTRACT

Purpose: Increasing evidence indicates that TGFbeta- and EGFR-signaling is involved in the pathogenesis of keratoacanthoma (KA) and squamous cell carcinoma (SCC) of the skin. We analyzed the expression pattern of TGFbeta-signaling components and screened for mutations in *tgfbetaR1*, *egfr*, *kras* and *braf* in KAs and SCCs.
Methods: Immunohistochemical analysis of TGFbeta1, TGFbetaR1, TGFbetaR2 and phospho-SMAD2/3 was performed on skin tumors (29 KAs, 30 well and 31 moderately differentiated SCCs). Mutation screening in hotspot regions of *tgfbetaR1*, *egfr*, *kras* and *braf* was performed through pyrosequencing of tumor DNA.
Findings: Expression of TGFbeta1, TGFbetaR1 and p-SMAD2/3 was increased in tumors as compared to surrounding skin. In KAs characteristic strong discontinuous membranous TGFbetaR1 expression pattern frequently associated with *kras* mutation was noted. SCCs showed continuous TGFbetaR1 expression, stronger p-SMAD2/3 expression and less frequent *kras* mutations. In tumors at sun-exposed sites stronger TGFbetaR1 expression was noted. One SCC showed *tgfbetaR1* mutation, but no other mutations were found.
Conclusion: Although *tgfbetaR1* germline mutations cause inherited KAs and our finding of strong discontinuous membranous expression in KAs suggests accumulation of functionally altered protein, we found no *tgfbetaR1* mutations or influence on TGFbeta-signaling, but frequent *kras* mutations in this subgroup of KAs. Characteristic TGFbetaR1 expression pattern in KA can facilitate histopathologic distinction from SCC.

Introduction

Keratoacanthomas (KAs) are fast growing tumors of the sun exposed skin, closely resembling squamous cell carcinomas (SCCs). After a growth phase of several weeks they typically become regressive and disappear without specific therapy. In contrast SCCs are clearly malignant tumors with local destructive growth and potential to metastasize. Histological distinction between the two tumor types is difficult, especially in well differentiated tumors. But immunohistochemical stainings are of limited value for differential diagnosis of these two entities. Several observations suggest a role of components of epidermal growth factor receptor (EGFR) and transforming growth factor beta (TGFbeta) pathways in tumorigenesis of both, KAs and SCCs: Immunohistochemical studies have shown overexpression of EGFR in KAs and SCCs of the skin [1] and recently treatment of patients with multiple and advanced KAs and SCCs with EGFR tyrosine kinase inhibitor erlotinib [2,3] and anti-EGFR antibody cetuximab [4,5] lead to clinical response. *Egfr* mutations were found in SCCs of head and neck [6–8], but the mechanisms of EGFR overexpression and accumulation in SCC of the skin remain unclear [9]. Members of the *ras* gene family (*hras*, *kras*, and *nras*) are part of intracellular EGFR signaling and primarily *hras* mutations have been found in KAs and SCCs [10]. Recently *kras*, *hras* and *nras* mutations in KAs and SCCs arising in melanoma patients under therapy with BRAF inhibitor vemurafenib have been reported [11]. This points to a role of intracellular signaling of EGFR in the pathogenesis of KAs and SCCs of the skin. As recently shown immunosuppressive drugs lead to elevated TGFbeta1 levels in patients after organ transplantation and increase the susceptibility for skin tumors like KA and SCC. Furthermore activation of TGFbeta signaling through SMAD2 is increased in normal skin, KAs and SCCs of transplant recipients compared to non-transplant

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http://dx.doi.org/10.1016/j.prp.2014.05.006
0344-0338/

recipients [12]. Recently germline mutations in *tgfbeta receptor 1* (*tgfbetaR1*) gene were identified in patients with multiple selfhealing squamous epithelioma (MSSE) or Fergusson-Smith disease, an inherited condition leading to acquisition of multiple KAs. These mutations lead to reduction or abrogation of TGFbeta signaling and subsequent SMAD signaling [13]. To further clarify the relation between TGFbeta signaling and EGFR pathway activation we analyzed components of TGFbeta signaling in sporadic KAs and SCCs of the skin and screened them for mutations in *tgfbetaR1* and components of the EGFR pathway. Differences in immunohistochemical or genetic phenotype between KAs and SCCs would be helpful in histopathological differential diagnosis of these tumor entities.

Materials and methods

Patients and skin samples

Paraffin material of 90 skin tumors cases (29 KAs, 30 well and 31 moderately differentiated SCCs), which were resected between 1993 and 2009, were retrieved from the files of the Institute of Pathology at the St. Vincentius Hospital Karlsruhe. The formalin-fixed and paraffin-embedded specimens were reevaluated by two experienced pathologists (A.D. and G.F.) using hematoxylin and eosin (HE) sections. Morphological features favoring KA were central horn-filled crater surrounded by overhanging "lips" of epithelium, abundant pale-staining cytoplasm of keratinocytes, tongues of atypical epithelial cells interposed between collagen bundles approximately parallel to the skin surface, intraepithelial abscesses often with acantholytic cells, lymphocytes and plasma cells in early lesions and neutrophils, eosinophils and histiocytes, sometimes with giant cells in later lesions. Criteria favoring SCC were predominantly endophytic growth without horn-filled crater, absence of epithelial "lips", little tendency to pale-staining cytoplasm of keratinocytes, absence of tumor tongues parallel to skin surface, acantholytic cells forming without associated neutrophils, absence of intraepithelial abscesses, individually necrotic keratinocytes (dyskeratotic cells) in growing lesion, detection of pseudoglandular formations, no prominent granulation tissue surrounding lesion, unless ulcerated, absence of granulomatous reaction and desmoplastic type of fibrosis, if present [14]. In SCCs grading into well and moderately differentiated tumors was performed. Well differentiated SCCs were characterized by minimal pleomorphism, occasional, usually basally located mitotic figures of squamous epithelium with obvious and often abundant keratinization. Moderately differentiated SCCs showed more structural disorganization with less obvious squamous epithelial derivation, moderate degree of nuclear pleomorphism, less keratin formation, often limited to keratin pearls, horn cysts or scattered individually keratinized cells and more frequent mitotic figures. For all cases a consensus diagnosis could be reached. Clinical data like tumor site, age and gender were obtained from the patients' files and given in Table 1. Approval for the analyses conducted in the study was received from the ethics committee at the Albert-Ludwigs-University of Freiburg (approval no. 324/09 of September 16th 2009).

Immunhistochemical analysis

Sections of 2 μm from the skin tumors and surrounding epidermis were cut onto silane-treated Super Frost slides (CML, Nemours, France) and left to dry overnight. The slides were deparaffinized in xylene and rehydrated in ethanol. After the heat induced epitope retrieval with citrate-buffer at pH6.0 for 1 h and cooling for 20 min, endogenous peroxidase was blocked using hydrogenperoxide 0.5% (Zytomed, Berlin, Germany) for 5 min. Antibodies against TGFbeta1 (mouse anti-human monoclonal, clone TGFB17, dilution 1:100, Novocastra, Newcastle upon Tyne, Great Britain), TGFbetaR1 (mouse anti-human monoclonal, clone 8A11, dilution 1:75, Novocastra, Newcastle upon Tyne, Great Britain), TGFbetaR2 (rabbit anti-human polyclonal, dilution 1:200, Zytomed, Berlin, Germany) and p-SMAD2/3 (goat anti-human polyclonal, dilution 1:200, Santa Cruz Biotechnology, Heidelberg, Germany) were incubated on sections for 1 h at room temperature. Appropriate secondary biotinylated antibody (PolyLink anti-mouse and anti-rabbit, DCS, Hamburg, Germany or Polylink anti-goat, Santa Cruz biotechnology, Heidelberg, Germany) was used at recommended dilution and incubated on sections for 20 min at room temperature. Thereafter peroxidase-label (horseradish peroxidase, DCS, Hamburg, Germany) was incubated for 20 min. Color was developed with chromogene substrate DAB (DCS, Hamburg, Germany) for 30 s, rinsed in tap water, counterstained with hematoxylin (MERCK KgaA, Darmstadt, Germany) for 1 min and dehydrated in graded alcohol and finally mounted with Pertex (Leica Microsystems, Wetzlar, Germany). Specificity of primary antibodies was ascertained as previously reported [15] and specimens processed without primary antibody were used as controls.

Cytoplasmatic (TGFbeta1), membranous (TGFbetaR1, TGFbetaR2) and nuclear (p-SMAD2/3) staining patterns in tumors and surrounding unaffected skin were evaluated using a 4 tier system from 0 (without expression), 1 (low expression), 2 (moderate expression) to 3 (strong expression) by two independent observers. Concordance between both observers was good (Cohen's kappa 0.303–0.620). All discordant cases were reviewed again and consensus score was achieved. If expression was heterogenous, then the assigned score was that observed in ≥75% of the epithelium. Pattern of membranous staining was further subdivided into continuous or discontinuous expression. Representative examples are shown in Fig. 1.

Mutation analysis

Sample preparation

Tumor areas marked by a pathologist (A.D.) on hematoxylin–eosin stained slides were manually microdissected on corresponding deparaffinised and hydrated slide samples and tumor tissue was collected in a microtube. After adding 180 μl Higuchi-buffer and 20 μl proteinase K samples were incubated overnight at 56 °C and finally denatured at 94 °C for 10 min.

PCR and pyrosequencing

Mutation analysis for *kras* exon 2 codons12, 13 and exon 3 codon61 was performed using Pyromark Q24 KRAS V2.0 (Qiagen, Hilden, Germany) according to manufacturer's protocol. *TgfbetaR1* mutation analysis of exon2 and 4 was performed as recently described [13]. For *egfr* exon18, 19 and 21 and *braf* exon15 five μl of tissue lysate was added to PCR mixture containing dNTPs (each 200 μM), 1.5 mM Magnesiumchloride, 50 mM KCl, 30 mM Tris–HCl, 0.2% Igepal®-CA630, Taq polymerase (1.25units; 5Prime, Hamburg, Germany), forward and reverse primers (400 nM each, see Table 2) and water to a final volume of 50 μl. PCR was conducted using following temperatures: 4 min at 95 °C, 35 cycles 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C followed by 7 min at 72 °C. For *egfr* nested PCR was performed using 5 μl of PCR product in second round PCR with corresponding primer (500 nM, see Table 2). 6–20 μl of PCR products were purified and denatured in vaccum preparation procedure using streptavidin-coupled sepharose beads as described in manufacturer' protocol (Qiagen, Hilden, Germany). Pyrosequencing was performed using corresponding sequencing primers (500 nM, see Table 2).

Statistics

Differences in immunohistochemical expression were determined using one-way ANOVA and univariate multifactorial